CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

21-602

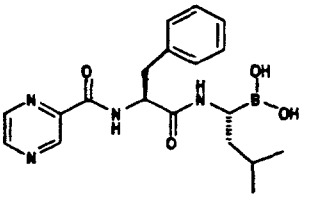
PHARMACOLOGY REVIEW

PHARMACOLOGY/TOXICOLOGY COVER SHEET

NDA number:	21-602
Review number:	1
Sequence number/date/type of submission:	RR2-001/1-21-03/NDA
Information to sponsor:	Yes (X) No ( )
Sponsor and/or agent:	Millennium Pharmaceuticals, Inc.
Manufacturer for drug substance:	Ash Stevens, 18655 Krause Street, Riverview, MI 48192
Reviewer name:	Lilliam A. Rosario, Ph.D.
Division name:	Division of Oncology Drug Products
HFD #:	150
Review completion date:	May 1, 2003

Drug:

Trade name:	VELCADE
Generic name (list alphabetically):	Bortezomib
Code name:	PS-341
Chemical name:	N-(2-pyrazinecarbonyl)-L-phenylalanine-L-leucine boronic acid
CAS registry number:	179324-69-7
Mole file number:	N/A
Molecular formula/molecular weight:	$C_{19}H_{25}BN_4O_4$ / 384.24
Structure:	



Relevant INDs/NDAs/DMFs:	IND [redacted]
Drug class:	Proteasome inhibitor
Indication:	Relapsed and refractory multiple myeloma
Clinical formulation:	Sterile lyophilized powder in single dose vial containing 3.5 bortezomib and 35 mg mannitol.
Route of administration:	Bolus intravenous injection
Proposed use:	The recommended dose of VELCADE is 1.3 mg/m^2/dose administered as a bolus intravenous injection twice weekly for two weeks (days 1, 4, 8, and 11) followed by a 10-day rest period (days 12-21).

Disclaimer: Tabular and graphical information is from sponsor's submission unless stated otherwise.

Executive Summary

I. Recommendations

A. Recommendation on Approvability: The non-clinical studies adequately support the use of PS-341 (Velcade) for the treatment of multiple myeloma patients.

B. Recommendation for Non-clinical Studies

1. Additional non-clinical studies appear warranted given the undefined etiology of the cardiovascular effects seen in multiple animal studies, as well as the occurrence of cardiovascular adverse events in patients.

- Given the narrow safety margin between the recommended clinical dose [redacted] and 100 % lethality in non-clinical studies (3.0 mg/m^2 in monkeys), we recommend the Sponsor determines the factors associated with PS-341 induced lethality at 12–14 hours post-dose.
- Since PS-341 promotes dissimilar effects in monkey and mouse, future studies should be conducted in a species that most closely models the human response.
- The Sponsor should identify the cardiac cell type(s) that are most effected following PS-341 administration to provide potential clinical interventions in the event of an overdose.
- Future non-clinical studies need to incorporate neuronal assessments to identify or rule out CNS involvement in these phenomena.

2. The Sponsor should conduct a study in cells transfected with a normal PrP gene to determine if administration of PS-341 results in the accumulation of proteins in the cytosol, similar to treatment with other proteasome inhibitors such as lactacystin or epoxomicin, as reported by Ma and Lindquist, 2002. Further, determine if misfolding of the normal PrP protein occurred with the formation of proteins with a PrP^{sc}-like conformation. The implications of these findings to the possible initiation and/or exacerbation of spongiform encephalopathies should be addressed.

C. Recommendations on Labeling

Refer to Appendix B

II. Summary of Nonclinical Findings

A. Brief Overview of Nonclinical Findings

PS-341 is a small, dipeptide boronic acid that reversibly inhibits the chymotrypsin-like proteolytic activity of the 20S-proteasome of mammalian cells. The 20S-proteasome binds with several regulatory proteins to create the 26S-proteasome complexes that hydrolyze proteins that have been marked for destruction by the ubiquitin enzyme cascade. Some evidence suggests that inhibition of the proteasome can act through multiple mechanisms leading to an arrest of cell growth. PS-341 inhibited cell growth and in some cases was cytotoxic for human tumor cells *in vitro*. In tumor xenograft models, PS-341 decreased tumor volume by up to 65%. The tumor content of PS-341 and relative tissue proteasome activity on these test sytems was generally not assessed.

The highest concentrations of radiolabeled PS-341 were found in the organs of metabolism and excretion (i.e., liver and kidneys) in rats and monkeys. The tissue:plasma concentration ratios in most tissues suggested rapid movement of labeled PS-341 from the vascular compartment into all tissues. Selected tissues (gastrointestinal tract, heart, kidneys, liver, lungs, and pancreas) and the carcass still contained administered radioactivity at 144 hours postdose after single IV administration. Radioactivity was detected in the brain of monkeys but not rats. The slow elimination and incomplete recovery of administered radioactivity in rats and cynomolgus monkeys are probably due to the extensive tissue distribution and retention of PS- 341 and metabolites. PS-341 was primarily metabolized via cytochrome P450 (3A4 and 2D6) and not via Phase II pathways e.g. glucuronidation and sulfation in monkeys and rats.

PS-341 did not induce the activities of CYP3A4 and 1A2 in primary cultured human hepatocytes and was a poor inhibitor of recombinant human CYP P450 isozymes 1A2, 2C9, 2C19, 2D6, and 3A4 with IC_{50} values of >18 μM (~7 μg/mL). These IC_{50} values are higher than the observed PS-341 C_{max} concentration seen in cancer patients (509 ng/mL (range=109-1300 ng/mL). Therefore, it is unlikely that PS-341 will change the metabolic clearance of concomitant medications. However, the potential increase or decrease in PS-341 activity by potent inducers or inhibitors of CYP3A4 and 2D6 has not been assessed. Further, PS-341 prevented the proteasome-dependent degradation of cytochrome P450 2E1 after ethanol induction. Other cytochrome P450 may also be degraded by proteasomes. Thus, PS-341 has the potential to modify the metabolism of a broad range of chemicals by increasing the intracellular concentration of cytochrome P450. This could possibly result in decreased exposure for drugs that are metabolized by cytochrome P450 with a concomitant decrease in efficacy or enhanced conversion of drug to activated forms.

Traditional toxicologic parameters, as well as neuropathological evaluations, toxicokinetic, and proteasome activity were assessed. Rodents were administered PS-341 as a single dose, weekly x 8, twice weekly for 2 and 26 weeks. In the 9-cycle 26-week rat study, PS-341-related mortality was observed at $\geq$0.9 mg/m^2 (day 50-day 197) and was due primarily to hematopoietic (bone marrow hypocellularity), gastrointestinal (hyperplasia and necrosis), and lymphoid system debilitation (lymphocytic depletion, atrophy, and necrosis of lymph nodes, spleen and thymus). Histopathological changes were observed in the heart (inflammation, hemorrhage, and necrosis),

liver (hypertrophy and necrosis), lung (necrosis and inflammation), kidney (necrosis and degeneration), sciatic nerve (necrosis), and spinal cord (inflammation); in general similar findings albeit with less severity were observed in scheduled deaths. Animals dosed $\geq 0.9 mg/m^2$ surviving to week 26 (end of treatment), exhibited multifocal neurotoxicity including brain dilatation, and degeneration of dorsal and ventral root ganglia, peripheral nerves, and spinal cord. Chronic progressive nephropathy was generally observed at 26 weeks at all doses; males appeared to be more susceptible to kidney changes. Histopathological changes in cardiac tissue included increased incidence of perivascular necrosis (at $\geq 0.6 mg/m^2$), myocardial degeneration, hemorrhage, and inflammation. Thrombocytopenia was observed at all PS-341 dose levels. Following the 8-week recovery period, myocardial and vascular inflammation, cardiac necrosis and chronic progressive nephropathy were still observed at all doses; the incidence of findings was not dose-dependent. There appeared to be some indication of reversibility of other findings at this time.

Monkeys were administered PS-341 as a single dose, for 24 hours, daily X 13 days, twice weekly for 2 weeks, and twice weekly for 4- and 13-three week cycles. In the 13-cycle monkey study, PS-341-related mortality was observed at dosages $\geq 0.9 mg/m^2$. The predominant findings in these animals were multifocal neurotoxicity (including brain necrosis and swelling, and degeneration of axons and myelin of dorsal root ganglia, peripheral nerves and spinal cord), severe anemia (bone marrow hypocellularity), thrombocytopenia, cardiotoxicity (necrosis, inflammation, and hemorrhage), and gastrointestinal intolerance (diffuse mucosal hyperplasia) and dehydration; in general similar findings albeit with less severity were observed in scheduled deaths. Neurotoxicity was documented as evidence of lack of neurological reflex observed at multiple sites at all dose levels in animals which survived to 38 weeks (end of dosing period). The incidence of histopathological findings demonstrating neurotoxicity was reduced following 8 weeks of recovery. The incidence of histopathological changes in cardiac tissue, including necrosis and inflammation was minimal and not dose dependent; the severity of cardiac findings was not reported. Kidney findings (hypertrophydegeneration, glomerulonephropathy, inflammation and the presence of hyaline casts) were observed at $\geq 0.9 mg/m^2$ PS-341; males appeared to be more susceptible to kidney changes. Lymphoid atrophy and/or necrosis was exhibited in thymus, spleen, lymph nodes and gut-associated lymphoid tissue. In addition, necrosis and atrophy of the gastrointestinal tract was observed in monkeys surviving to 38 weeks.

In monkeys and rodents there exists only a small dose margin between lethality (at doses $\geq 0.9 mg/m^2$) and the maximum tolerated dose or HNSTD ($0.6 mg/m^2$ PS-341). It is noteworthy that the lethal dose and maximum tolerated dose are the same in both species. Furthermore, there is no safety margin compared to the proposed clinical dose ($1.3 mg/m^2$). Cardiac changes were observed in rodents and monkeys; these findings were not dose-dependent. In rodents, cardiac findings did not appear to reverse after 8-weeks post drug administration; severity of cardiac findings was not reported. Monkeys and rats were dosed with a similar schedule as recommended for patients (twice weekly dosing for 2 weeks followed by 1-week rest). Neurotoxicity exhibited in monkeys and rodent was multifocal and included nerve degeneration of dorsal root ganglia, peripheral nerves and spinal cord at $\geq 0.6 mg/m^2$ (one half the recommended clinical dose of $1.3 mg/m^2$); monkeys appeared to be more susceptible to the neurotoxic effects of PS-341 compared to rodents. Neurotoxicity continued to be exhibited following recovery periods in monkeys and rodents. Clinical observations of tremors and reduced motor activity were exhibited in monkeys;

rodents also exhibited reduced motor activity. Both species exhibited hematopoietic, gastrointestinal and lymphoid system debilitation and glomerulonephropathy.

Dose- and schedule-dependent changes in AUC and C_{max} were also exhibited in both species. Drug exposure with increasing dose was more linear in monkeys compared to rodents; the explanation for this difference is not known. After multiple doses of PS-341, there was a decrease in clearance that resulted in an increase in the terminal elimination half-life ($t_{1/2}$) and AUC (3-4 fold) in rats and cynomolgus monkeys, suggesting drug accumulation. An increase in half-life and AUC and decrease in clearance were also observed in solid tumor patients. Female decedent rats appeared to exhibit a greater degree of toxicity as compared to males. However, pharmacokinetic data were similar between gender. Using an ———— assay to measure inhibition of the chymotrypsin-like proteolytic activity in white blood cells, it was shown that inhibition of proteasome activity increased with dose and recovered in about 72 hours in rats, cynomolgus monkeys, and multiple myeloma patients. The tissue distribution, steep dose response curve, and the spectrum of toxicities suggest that PS-341 may cause toxicity through mechanisms other than solely by inhibition of the 20S-proteasome. PS-341 is a substituted dipeptide, and as such, may interact with other cellular sites.

Cardiovascular safety pharmacology studies conducted in cynomolgus monkeys showed that administration of dosages $\geq$ 3.0 mg/m^2 PS-341 (twice the recommended clinical dose) resulted in initial physiologically significant heart rate elevations, which preceded a profound progressive hypotension, bradycardia, and death 12-14 hours postdose. Follow-up studies in anesthetized monkeys showed PS-341 increased heart rate ($\geq$1.2 mg/m^2), decreased mean arterial pressure ($\geq$2.4 mg/m^2), increased ventricular contractility ($\geq$3.6 mg/m^2), and increased cardiac output ($\geq$3.6 mg/m^2). Mortality was not reported in this study, however, this study is inadequate to address issues of drug-associated mortality observed in the previous studies because monkeys were sacrificed 6 hours post drug administration, before signs of terminal hypotension and imminent mortality were manifested. It appears that changes in cardiovascular parameters reflect pharmacodynamic effects of PS-341 that are not directly related to plasma concentration, but instead may be dependent upon intracellular or 'target bound' kinetics. Moreover, PS-341 and metabolites have been shown to be sequestered in the myocardium. Cardiac necrosis was observed following repeated dosing at 1.2 mg/m^2, further suggesting that PS-341-induced cardiac effects may be dependent on or explained by the local disposition of the drug.

To further investigate the possible mechanisms of cardiac changes, PS-341 was administered to mice. The same PS-341 induced-sequelae of cardiac events were not observed in mice and monkeys. Further, given the disparity in lethal doses in monkeys and mice ($\geq$3.0 mg/m^2 versus 30 mg/m^2, respectively) and the lack of data on drug distribution and exposure in mice, it is unclear whether the mouse is an appropriate species in which to investigate PS-341-induced cardiovascular effects.

Additional non-clinical studies appear warranted given the undefined etiology of the cardiovascular effects seen in multiple animal studies, as well as the occurrence of cardiovascular adverse events in patients. Given the narrow safety margin between the recommended clinical dose (1.2 mg/m^2) and 100 % lethality in non-clinical studies (3.0 mg/m^2 in monkeys), we recommend the sponsor determines the factors associated with PS-341 induced lethality at 12–14

hours post-dose. Since PS-341 promotes dissimilar effects in monkey and mouse, future studies should be conducted in an appropriate species that most closely model the human response. The Sponsor should identify the cardiac cell type(s) that are most effected following PS-341 administration to provide potential clinical interventions in the event of an overdose. Future non-clinical studies need to incorporate neuronal assessments to identify or rule out CNS involvement in these phenomena.

PS-341 was positive for clastogenic activity (structural chromosomal aberrations) in the *in vitro* [redacted] assay using [redacted] PS-341 was not genotoxic when tested in the [redacted] assay [redacted] and [redacted] assay in [redacted]

Teratological effects of PS-341 were examined in both the rat and rabbit. No formal evaluation of effects on fertility or peri-and postnatal development (Segments I and III, respectively) were conducted. Nonetheless, pregnant rabbits given PS-341 during organogenesis at a dose of 0.6 mg/m^2 experienced significant post-implantation loss and decreased number of live fetuses at minimally maternal toxic doses. Live fetuses from these litters also showed significant decreases in fetal weight. The dose is approximately 0.5 times the clinical dose of 1.3 mg/m^2 based on body surface area. Based on embryolethality findings in rats and rabbit, as well as the effects of PS-341 on primary and secondary sex organs as observed in the 6-month rat study and the 9-month monkey toxicity study, PS-341 is likely to have a potential negative or adverse effect on pregnancy. However, PS-341 was not teratogenic in rats and rabbits at the highest dose tested (0.075 mg/kg; 0.5mg/m^2 in the rat and 0.05 mg/kg; 0.6 mg/m^2 in the rabbit) when administered during organogenesis. These dosages are approximately half the clinical dose of 1.3 mg/m^2 based on body surface area.

A tissue irritation study was conducted in male New Zealand white rabbits after administration of 1.2 mg/m^2 PS-341 as a single intravenous, perivenous, intramuscular, or subcutaneous injection. PS-341was considered a tissue irritant when administered by the perivenous and intramuscular routes and no tissue reaction was seen after subcutaneous and intravenous administration.

A study utilizing intra-prostatic administration of PS341 was conducted in dogs. Mild to severe progressive clinical signs consistent with peripheral neuropathy were observed. Most notable is the apparent retrograde degeneration from the neural plexus of the prostate to the ventral roots of the spinal cord (Wallerian degeneration). The same type of lesion has been observed in 4-week and 38-week cynomolgus monkey toxicity studies (IV dosing). These data suggest that extravascular extravasation or, local tissue injection of PS-341 in highly innervated tissues, may be associated with particularly severe and progressive neuronal injury.

Limited evidence (Ma and Lindquist, 2002) suggests the possibility that inhibition of the proteasome could increase the concentration of prion proteins (PrP) in the cytosol of neurons with the formation of proteins with a PrP^{sc}-like conformation, a misfolded protein that has a predominantly β-sheet secondary structure. It has been postulated that PrP^{sc} is the transmissible agent or the predominant part of that agent in a fatal neuro-degenerative disease in sheep and goats that manifests as a spongiform encephalopathy; analogs in other species include cattle (mad cow disease or bovine spongiform encephalophy) and humans (Creutzfeldt-Jakob disease). This

protein may act as a chaperone protein that is able to refold PrP^{c} to PrP^{sc}, thus propagating itself and the disease.

The Sponsor should conduct a similar study to determine if administration of PS-341 results in the accumulation of proteins in the cytosol, similar to treatment with other proteasome inhibitors such as lactacystin or epoxomicin, as reported by Ma and Lindquist, 2002. Further, determine if misfolding of the normal PrP protein occurred with the formation of proteins with a PrP^{sc}-like conformation. The implications of these findings to the possible initiation and/or exacerbation of spongiform encephalopathies should be addressed.

B. Pharmacology

PS-341 is a small, dipeptide boronic acid that reversibly inhibits the chymotrypsin-like proteolytic activity of the 20S-proteasome of mammalian cells. The molecule diffuses freely across the cell membrane and binds to the proteasome at significantly lower concentrations than it does to a number of other proteases. The 20S-proteasome binds with several regulatory proteins to create the 26S-proteasome complexes that hydrolyzes proteins that have been marked for destruction by the ubiquitin enzyme cascade. This ubiquitin-proteasome system is responsible for essential elements of homeostatic control within the cell in G_0 and numerous processes through the course of the cell cycle. It is hypothesized that when PS-341 inhibits this system, the cell cycle arrests at the transition G_2-M, leading inhibited cells to initiate apoptosis. It is also hypothesized this system activates NFαB by inhibition of proteasome-mediated IκB degradation that, in turn can make cells more sensitive to induction of apoptosis. Thus, some evidence suggests that inhibition of the proteasome can act through multiple mechanisms leading to an arrest of cell growth.

C. Nonclinical Safety Issues Relevant to Clinical Use

Safety Margin compared to recommended clinical dose:
In monkeys and rodents there exists only a small dose margin between lethality at doses $\geq 0.9 mg/m^2$ and the maximum tolerated dose or HNSTD of $0.6 mg/m^2$ PS-341. It is noteworthy that the lethal dose and maximum tolerated dose are the same in both species. Furthermore, there is no safety margin compared to the proposed clinical dose (1.3 mg/m^2).

Species	Study[A]	Lethal dose (mg/m^2)	MTD (mg/m^2)	Safety Margin
Rat	9-cycles (6-months)	≥0.9	0.6	<1
Monkey	13-cycles (9-months)	≥0.9	0.6	<1
Human			1.3[A,B]	

[A] Twice weekly schedule
[B] Recommended dose

Cardiotoxicity:
Cardiovascular safety pharmacology studies conducted in cynomolgus monkeys showed that administration of dosages $\geq$ 3.0 mg/m^2 PS-341 (twice the recommended clinical dose) resulted in initial physiologically significant heart rate elevations, which preceded a profound progressive hypotension, bradycardia, and death 12-14 hours postdose. Increased heart rate and decreased mean arterial pressure were also observed at lower doses of PS-341 ($\geq$1.2 mg/m^2 and $\geq$2.4 mg/m^2, respectively).

It appears that changes in cardiovascular parameters reflect pharmacodynamic effects of PS-341 that are not directly related to plasma concentration, but instead may be dependent upon intracellular or 'target bound' kinetics. Moreover, PS-341 and metaboliets have been shown to be sequestered in the myocardium. Cardiac necrosis was observed following repeated dosing at 1.2 mg/m^2, further suggesting that PS-341-induced cardiac effects may be dependent on or explained by the local disposition of the drug.

These data on the cardiovascular effects of PS-341 indicate that acutely lethal IV dosages of PS-341 are associated with increases in heart rate, decreased mean arterial pressure, and ultimately terminal hypotension.

Intraprotstatic Injection of PS-341:
Intraprostatic administration of PS-341 resulted in severe neurological dysfunction following single (12 mg/m^2) or repeat-dose (2.6 mg/m^2; bi-weekly for 3 doses) administration. Most notable is the apparent retrograde degeneration from the neural plexus of the prostate to the ventral roots of the spinal cord (Wallerien degeneration) after local intra-prostatic administration of PS-341. The same type of lesion has been observed in 4-week and 38-week cynomolgus monkey toxicity studies (IV dosing). These data suggest that extravascular extravasation or, local tissue injection of PS-341 in highly innervated tissues, may be associated with particularly severe and progressive neuronal injury.

Neuropathy
Monkeys and rats were dosed for 13-three weeks with a similar schedule as recommended for patients (twice weekly dosing for 2 weeks followed by 1-week rest). Neurotoxicity was multifocal and included brain necrosis, and swelling and degeneration of axons and myelin of dorsal root ganglia, peripheral nerves, and spinal cord. Multifocal nerve degeneration of dorsal root ganglia, peripheral nerves and spinal cord was observed at $\geq$0.6 mg/m^2 (one half the recommended clinical dose of 1.3 mg/m^2).

Cytochrome P450 Regulation
PS-341 prevented the proteasome-dependent degradation of cytochrome P450 2E1 after ethanol induction. This degradation returns intracellular expression of P450 2E1 to constitutive concentrations after induction. Other cytochromes P450 may also be degraded by proteasomes. Thus, PS-341 has the potential to modify the metabolism of a broad range of chemicals by increasing the intracellular concentration of cytochrome P450. This could possibly result in decreased exposure for drugs that are metabolized by

cytochrome P450 with a concomitant decrease in efficacy or enhanced conversion of drug to activated forms.

Prion Proteins

Limited evidence suggests the possibility that inhibition of the proteasome could increase the concentration of prion proteins (PrP) in the cytosol of neurons. Normal PrP (PrP^c) undergoes post-translational modifications including folding into a mostly α-helical secondary structure. As much as 10% of PrP^c is processed incorrectly, resulting in its diversion to the ubiquitin-proteasome-system. One malformation of PrP is the formation of PrP^{sc}, a misfolded protein that has a predominantly β-sheet secondary structure. It has been postulated that PrP^{sc} is the transmissible agent or the predominant part of that agent in a fatal neuro-degenerative disease in sheep and goats that manifests as a spongiform encephalopathy; analogs in other species include cattle (mad cow disease or bovine spongiform encephalophy) and humans (Creutzfeldt-Jakob disease). This protein may act as a chaperone protein that is able to refold PrP^c to PrP^{sc}, thus propagating itself and the disease.

It has been shown that treatment of cells transfected with a normal PrP gene with other proteasome inhibitors, such as lactacystin or epoxomicin, results in the accumulation of proteins in the cytosol (Ma and Lindquist, 2002). The concentrations of these proteins, except for PrP^c, return to normal after the inhibition has been removed. Misfolding of the normal PrP protein occurred with the formation of proteins with a PrP^{sc}-like conformation. One possible implication suggest that inhibition of the proteasome pathway could lead to the accumulation of PrP protein followed by the formation of PrP^{sc} -like β-sheet proteins that could refold yet more PrP proteins. It is conceivable that this could exacerbate spongiform encephalopathies or even initiate them by inducing the formation of PrP^{sc} proteins. PS-341 is found in relatively low concentrations in the brains of animals given a single radiolabeled dose. Nonetheless, after chronic administration, brain necrosis was observed suggesting that PS-341 crosses the blood brain barrier. In talks with the Agency, the Sponsor offered to repeat the above-mentioned studies using PS-341 as the inhibitor, however, this information was not provided with the current NDA.

Microarray Data

In the review of supporting materials for this application, it was noted to the Applicant that they had not included in their package a reference from the "open literature" that used complementary whole genome technologies in yeast to investigate their proposed mechanisms of action. In their response, the applicant stated that this publication was not included in the current submission because they were not sure as to the relevance of the findings in yeast cells to the higher eukaryotic cells. Moreover, they added that they have initiated a

As a justification for not reporting this information, the Applicant noted that Dr. Steve Galson proposed a 'safe harbor' process for evaluation of pharmacogenomic data at an FDA/Industry forum on Pharmacogenetics and Pharmacogenomics in May 2002. We have requested clarification from Dr. Woodcock on whether the applicant is required to submit this data, albeit not to base a regulatory action. Since it has been proposed that the "safe harbor" mechanism will "help the Agency and industry learn from these data" (Dr. Galson, Pink Sheet March 17, 2003), we would also like to know whether we can request the Applicant to submit the data. An action on this NDA is expected in the near future, we do not expect the data to be available prior to that time.

III. Administrative

A. Reviewer signature: Lilliam A. Rosario, Ph.D.

B. Supervisor signature: Concurrence - David Morse, Ph.D.

Non-Concurrence - _____
(see memo attached)

C. cc: list: bradelys, leightonj, brossp, kaner, farrella

APPEARS THIS WAY
ON ORIGINAL

TABLE OF CONTENTS - PHARMACOLOGY/TOXICOLOGY REVIEW

I. PHARMACOLOGY:

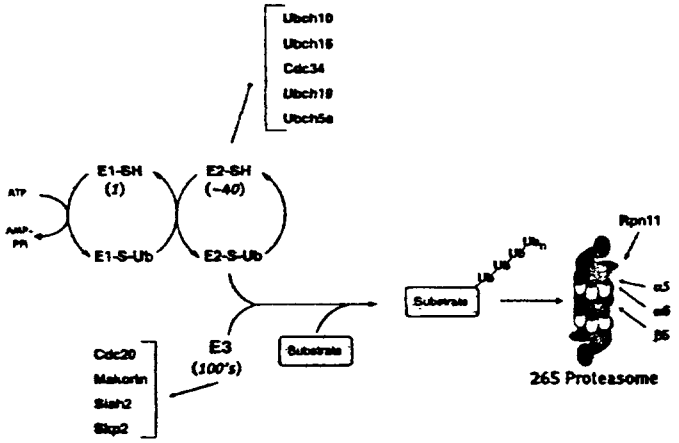
Reviewed by W. David McGuinn, Jr., Ph. D., D.A.B.T.

THE PHARMACOLOGY OF VELCADE

The Biochemistry of the Proteasome.

The importance of protein synthesis in the regulation of cell function and reproduction is among the oldest concepts in biochemistry but investigators began to appreciate the importance of protein degradation in cell regulation only in the 1970's (AL Goldberg and AC St. John, 1976, *Annu. Rev. Biochem.* **45**;747-803). More recently the biochemical sciences have begun to characterize the ubiquitin-proteasome pathway; the mechanism of most regulated protein degradation.

In this pathway, proteins destined for degradation are first marked by the covalent addition of multiple molecules of ubiquitin. Three distinct enzymes participate sequentially in this polyubiquitination, an ATP-dependent ubiquitin-activation enzyme (E1), an ubiquitin-conjugation enzyme (E2), and an ubiquitin-protein ligase (E3) (AL Hass *et al* 1982, *J Biol Chem* Mar 10;257(5):2543-8). This sequential processing links the C-terminus of ubiquitin to specific sites on the target protein, particularly the ε-amino group of lysine. Protein bound ubiquitin itself can be linked by ubiquitin at its lysine 48 residue, forming a long polyubiquitin chain (Z Chen and CM Pickart, 1990, *J Biol Chem*, Dec 15;**265**(35):21835-42). The ubiquitinated protein is then recognized and degraded by the proteasome-complex, a large (2,100 kD) multi-unit protease with numerous substrate binding sites and specificities for numerous amino-acid sequences (CM Pickert, 2001, *Annu Rev Biochem*; **70**:503-33). The following schematic shows the polyubiquitination reaction and lists some of the genes that encode for the enzymes involved.



The heart of the proteasome-complex is the 20 Svedberg (20S) proteasome itself, about 700 kD in molecular weight. In eukaryotic cells, 14 genes code for the 14 distinct proteins of this enzyme. Seven of these proteins aggregate to form the α-subunit and seven to form the β-

subunit (P Zwickl *et al.* 1992, *Biochemistry*, Feb 4;31(4):964-72). Each of the heptameric subunits is annular. Four subunits stack to form a cylinder with α-subunits at each end and two β-subunits in the center (W Baumeister *et al*, 1998, *Cell*, 923;67-380). Thus, this stack is a multi-gene product dimer with a 72-point group symmetry (J Lowe et al, 1995, *Science* Apr 28;268(5210):533-9). The 20S-proteasome has multiple proteolytic sites on the interior wall of the hollow cylinder. Independent of its regulatory proteins (below) the 20S-proteasome is probably completely inactive *in vivo*. The N-terminal portions of the α-subunits obstruct the opening to the interior cavity of the cylinder. *In vitro*, the 20S proteasome can hydrolyze only completely denatured short peptide chains and this only after it has been 'activated' by low concentrations of SDS. The SDS probably changes the conformation of the N-terminal portion of the α-subunit and allows entry of the protein substrates into the interior of the cylinder. This proteolysis of small peptides in the presence of SDS is used to assay tissue isolates for proteasome activity. The 20S-proteasome occurs in high copy number within the cell and is easily purified and quantified. (P Zwickl, *Curr Top Microbiol Immunol.* 2002;268:23-41).

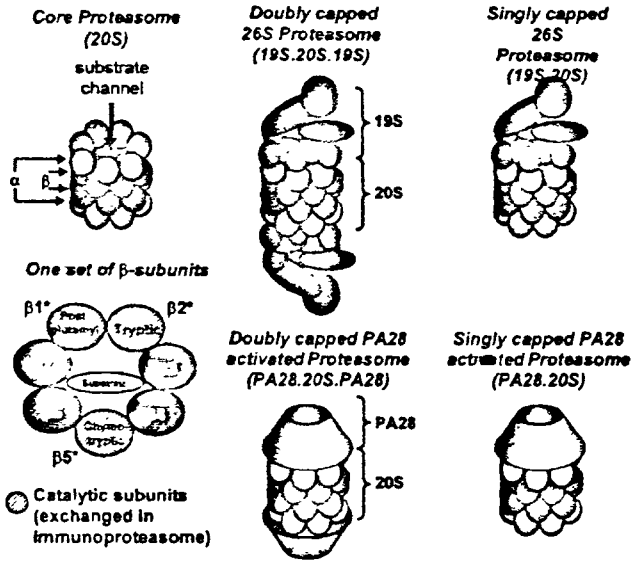
In higher eukaryotes, three of the seven proteins that make up a β-subunit are catalytic, resulting in at least six catalytic sites per 20S proteasome. These catalytic proteins are known as β1, β2 and β5. The function of the other four proteins is unknown but they probably control substrate binding and movement through the proteasome. Among the better characterized proteolytic activities of the proteasome are a chymotrypsin-like hydrolysis that cleaves proteins at tyrosine or phenylalanine residues; a trypsin like activity that acts at arginine or lysine sites; and a postglutamyl hydrolysis that cleaves at glutamate or other acidic residues. Within the eukaryotic cell, the β1, β2 and β5 have close protein homologues known as LMP2, MECL-1 and LMP7 respectively (W Baumeister et al, 1998, *Cell*, 923;67-380). The expression of these homologous proteins is inducible by a variety of external conditions. One such condition is treatment with γ-interferon (K Fruh *et al*, 1994, *EMBO J*, 13;3236-3244). When a cell is induced to expresses these homologous proteins, it incorporates them into newly formed proteasomes. These proteasome isoforms are sometimes referred to as immuno-proteasomes or γ-interferon proteasomes. The presence of these homologues modifies the proteasome substrate specificity. Nevertheless, proteasomes with either set of proteins, constitutive or inducible, degrade large ubiquitin labeled proteins at about the same rate. The working hypothesis to explain the role of the inducible proteins is that they are transiently expressed to regulate specific functions of the cell such as antigen processing or the cell cycle.

As mentioned above, the 20S proteasome is probably not catalytically active on its own *in vivo*. It must first bind to various other proteins known as activators to form proteasome-activator complexes before it can hydrolyze ubiquitin-bound protein substrates. Different activators can bind to form different proteasome-activator complexes, each with different substrate specificity. One of these proteasome-activator proteins, PA700 (or the 19S cap), is a 20S subunit, 700 kD complex that can bind at the opening of one or both of the α-subunits of the 20S proteasome (GM Adams *et al.* 1997, *J Mol Biol* Oct 31;273(3):646-57, and GM Adams *et al.*, 1998, *Biochemistry* 1998 Sep 15;37(37):12927-32). This proteasome-activator complex containing two PA700 subunits has a molecular weight of about 2,100 kD and a sedimentation coefficient of 26S, hence its moniker, the 26S proteasome. The binding of the PA700 activator to the 20S proteasome to form the 26S proteasome requires ATP hydrolysis, implying covalent binding between the two units. The binding of the PA700 subunits at the ends of the α-subunits of the 20S-proteasome implies that these proteins serve as gatekeepers, changing the conformation of the opening in the α-subunit and facilitating the entry of doomed proteins. The

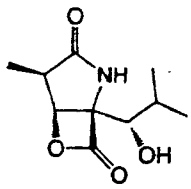
PA700 subunit probably also serves to unfold and denature large proteins to a simple linear peptide chain before they enter the 20S proteasome subunit for processing. The 26S proteasome catalyzes proteolysis of large polyubiquinated proteins rapidly but the process consumes ATP continuously. Even if proteolysis within the 20S-proteasome subunit is a passive process, one would expect the purposeful movement of a large protein chain through the narrow 13 Angstrom annular space of the 20S subunit to be active and energy intensive.
Unlike other proteases, the PA700 subunit does not recognize specific amino acid sequences but rather interacts with the ubiquitin bound to targeted proteins. Thus the specificity of the proteasome is not for particular large proteins but for any large ubiquitin-tagged protein and the determination of which proteins are to be processed by the proteasome is made not by the proteasome itself but by the enzymes of the ubiquitin cascade described above. The PA700 subunit has at least one other function – it removes the ubiquitin tags from the protein being degraded by the 20S proteasome. This process is probably essential since the ubiquitin tags almost certainly would obstruct the movement of the protein chain through the annular space of the proteasome (M Groll and R Huber, 2003, *Int J Biochem Cell Biol*, May;35(5):606-16).

The function of the 20S proteasome can also be modulated by interaction with a protein called PA28 (or the 11S regulator) (CW Gray et al, 1994, *J Mol Biol* 1994 Feb 11;**236**(1):7-15). This protein contains two subunits, α and β, each weighing about 28 kD. The subunits are quite different, with only 50% structural similarity. Either six or seven (this is currently uncertain) of these subunits link to form a large annular protein that weighs about 180 kD. Like the PA700 subunit, one or two PA28 regulator proteins bind to the α-subunits of the 20S proteasome. Unlike PA700, this process does not require ATP. Large ubiquitin-bound proteins are not substrates for this PA28-activated proteasome. Instead, it efficiently hydrolyzes short peptides. This complex may further process the products of 26S-proteasome proteolysis. Nevertheless, this activity is inducible by γ-interferon. Some investigators have postulated that this proteasome may be involved in the processing of small peptides for presentation by class I major histocompatibility complexes (MP Belich and J Trowsdale, 1995, *Mol Biol Rep*, **21**(1):53-6). One PA28 regulator protein can also bind one end of the 20S proteasome while one molecule of the PA700 binds the other. This combination further changes the substrate specificity of the proteasome. Much work remains to characterize the function of these varied proteasome activities.

The following schematic diagram shows the presumed arrangement of the various proteasome isoforms.



In 1991 Omura *et al.* (1991, J. Antibiot. 44:113-118) reported the discovery of a peptidomemetic metabolite of streptomyces called lactacystin that induced differentiation in neuronal cells *in vitro*. G. Fenteany and coworkers (1995, Science, **268**:726-731) then demonstrated that this biological activity was associated with the inhibition of the function of the 20S proteasome by lactacystin. L. R. Dick *et al.* subsequently presented evidence that lactacystin hydrolyzes in aqueous solution to clasto-lactacystin β-lactone, the ultimate inhibitor of the 20S proteasome (1996, *J. Biol. Chem.* **271**(13):7273-7276). Other natural products, including lactacystin, MG-132, PSI, and epoximicin can inhibit proteasome activity. These inhibitors probably act by preventing access to the interior catalytic site of the proteasome. These discoveries and others sparked a search for molecules that could inhibit the 20S proteasome.

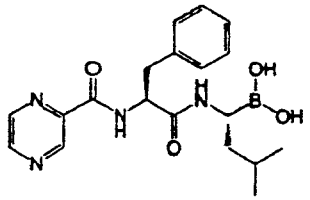


***clasto*-Lactacystin**
β-Lactone

Mechanism of action of PS-341

PS-341 is a small, dipeptide boronic acid that inhibits the proteasome by binding reversibly at a yet unknown amino acid sequence. The molecule diffuses freely across the cell membrane. It binds to the proteosome at significantly lower concentrations than it does to a number of other proteases. Millennium Pharmaceuticals, the sponsor of IND [redacted] and NDA

21602, is developing PS-341 to treat human malignancies. They have demonstrated that PS-341 is cytotoxic, growth inhibitory and has antitumor activities in several *in vitro* and *in vivo* assay systems.



PS-341

Proteins essential for cellular division, the expression of which is regulated by the ubiquitin-proteasome system, include cyclins, cyclin dependent kinase inhibitors, NF-κB and p53 (CJ Sherr, 1996, *Science*, Dec 6;274(5293):1672-7). By inhibiting proteasome activity and thus the destruction of various proteins involved in the cell cycle, PS-341 disrupts normal cell division, blocking it at G_2-M. Because of this block, the cell is forced to initiate apoptosis (YH Ling *et al*, 2003, *Clin Cancer Res*, Mar;9(3):1145-54). The ubiquitin-proteasome system may also control the intracellular concentrations of cell adhesion molecules such as E-selectin, ICAM-1, and VCAM-1, which are involved in tumor metastasis and angiogenesis. The expression of these molecules is controlled by nuclear factor-κB (NF-κB) (MA Read *et al.*, 1995, *Immunity*, May;2(5):493-506), the ultimate expression of which is regulated by proteasome destruction. Thus, the ubiquitin-proteasome may influence neoplastic growth and metastasis by down-regulation of NF-κB dependent cell adhesion molecule expression. In addition, NF-κB is also transcriptionally upregulated by ubiquitin-proteasome dependent degradation of the inhibitor protein IκB. The activation of NF-κB appears to suppress the signals for cell death (Chu *et al.*, 1997), while inhibiting NFκB activation by inhibition of proteasome-mediated IκB degradation can make cells more sensitive to induction of apoptosis (Beg, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). Thus, some evidence suggests that inhibition of the proteasome can act through multiple mechanisms leading to an arrest of tumor growth and possibly metastasis and angiogenesis.

F. Bardag-Gorce *et al.* (2002, *Free Radic Biol Med* Jan 1;32(1):17-21) have shown that cytochrome P450 2E1 is degraded by proteasomes after ethanol induction, thus returning intracellular expression to constitutive concentrations. PS-341 prevented this degradation. Other cytochromes P450 may also be degraded by proteasomes after induction (KK Korsmeyer *et al.*, *Arch Biochem Biophys* 1999 May 1;365(1):31-44). Thus, PS-341 has the potential to modify the metabolism of a broad range of chemicals by increasing the intracellular concentration of cytochrome P450. This could possibly result in decreased exposure for drugs that are metabolized by cytochrome P450 with a concomitant decrease in efficacy.

NCI has shown that PS-341 inhibits cell growth and in some cases is cytotoxic for human tumor cell types using the *in vitro* cancer screen against a panel of 60 human cancer cell lines. The average GI_{50} of PS-341 across the 60 cell lines was 3.8 nM. NCI also assessed PS-341 in the hollow-fiber screening program and found it active in both intraperitoneal and subcutaneous implanted hollow fiber/tumor cells. In both the HT-29 human colon and PC-3 human prostate tumor xenograft model in athymic mice, PS-341 given IV weekly for 4 weeks (0.3 or 1.0 mg/kg/dose) decreased tumor volume by up to 50% and 65% in the high dose group, for HT-29 and PC-3 xenografts, respectively.

Inhibition of proteasomes and Prion disease

Many proteins synthesized on ribosomes undergo post-translational modifications in the lumen of the endoplasmic reticulum (ER). These modifications include conformational changes enforced by chaperone proteins and the removal or addition of functional groups by other enzymes. Sometimes this process goes awry and proteins are misfolded or mismodified. Such malformed proteins are then transported to the cytosol where they are marked for destruction by the ubiquitin system then degraded by the proteasome.

Prion protein (PrP) is a found on the surface of many cells, but neurons have the highest copy numbers. This protein is probably multifunctional. Evidence implicates its involvement in the transport and metabolism of metal-ions associated with protection against oxidative stress, in signal transduction and possibly even as a nucleic acid chaperone protein (EA Derrinton and JL Darlix, *Drug News Perspect.* 2002 May; **15**(4):206-219). Normal PrP (PrP^c) undergoes post-translational modifications that include folding into a mostly α-helical secondary structure, the addition of glycosyl-phosphatidylinositol anchor and glycosylation. As much as 10% of PrP^c is processed incorrectly, resulting in its diversion to the ubiquitin-proteasome-system (J Ma and S Lindquist, 1999, *Proc. Natl. Acad. Sci. U. S. A.* **98**:14955-14960). Mutations can increase this percentage.

One malformation of PrP is the formation of PrP^{sc}, a misfolded protein that has a predominantly β-sheet secondary structure. The sc in the name PrP^{sc} stands for scrapie, a transmissible always fatal neuro-degenerative disease in sheep and goats (C Ersdal *et al.*, 2003 *Vet Pathol*, Mar, **40**(2):164-174). The disease manifests as a spongiform encephalopathy and is thought to have analogs in other species including cattle (mad cow disease or bovine spongiform encephalophy) and humans (Creutzfeldt-Jakob disease). S. B Prusiner (*Proc. Natl. Acad. Sci. U. S. A.*, 1998, 95:13363) and others have postulated that PrP^{sc} is the transmissible agent or the predominant part of that agent in these diseases. This protein may act as a chaperone protein that is able to refold PrP^c to PrP^{sc}, thus propagating itself and the disease.

J Ma and S Lindquist (2002, *Science*, **298**;1785-1788) have observed that some proteins accumulate in the cytosol of cells transfected with a normal PrP gene and treated with proteasome inhibitors such as lactacystin or epoxomicin. The concentrations of most of these proteins returns to normal after the inhibition has been removed. But, that of PrP^c does not, the concentrations remains high after the removal of the inhibition. This process is cytotoxic and could be the mechanism of the neurotoxicity observed with PS-341 clinically. A more worrisome observation in this study was that after the investigators removed the inhibition of the proteasome, misfolding of the normal PrP protein occurred with the formation of proteins with a PrP^{sc}-like conformation.

The implication of this work is that inhibition of the proteasome pathway could lead to the accumulation of PrP protein followed by the formation of scrapie like β-sheet proteins that could refold yet more PrP proteins. The process could become self-perpetuating and could spread among cells of the nervous system resulting in a spongiform encephalopathy. Such a disease would probably not be easily transmissible from one person to another but it would be fatal to the patient in which it developed. The possibility of this process occurring may be greater in patients with mutations in their PrP gene. Mutations in the PrP gene are observed in familial Creutzfeldt-Jakob disease. Other neurodegenerative disorders including Alzheimer's, Huntington's, Parkinson's and cystic fibrosis involve the formation of aggregates of misfolded proteins. Any potential effect of PS-341 on these diseases is unknown.

I recollect that in talks with the NCI, the sponsor [redacted] I did not find such a study among the sponsor's submissions.

Review of submitted studies.

1) Kinetic evaluation of PS-341, PS-273 and PS-304. RPT-00102. Module 4. Folder 4211-prim-pd, file158.pdf.

T Soucy and E Lightcap of Millennium Pharmaceuticals designed this experiment to determine the rate constants for the binding of PS-341, PS-273 and PS-304 to the 20S proteasome of rabbit reticulocytes. The method of their analysis is somewhat unorthodox, as is some of their terminology. They claim that standard Michaelis-Menten analysis is inappropriate. I have not confirmed the validity of their analysis. The report is sparsely referenced and it omits considerable detail.

Stein *et al.* originally developed this enzyme assay in 1996 (*Biochemistry*, 35, 3899-3908). In the experiments of their 'Association Method' the authors of the current report added purified 20S proteasome to HEPES-buffer (pH 8.0) containing 0.035% SDS. As mentioned above, SDS is necessary to activate the proteasome absent protein activators. To this, they added 10 μM of the polypeptide substrate Suc-Leu-Leu-Val-Tyr-AMC, where AMC is adriamycin. This peptide is a standard for determining chymotrypsin like activity. The proteasome cleaves the adriamycin, which once free becomes fluorescent. The increase in fluorescence marks the progress of the reaction. The authors then fit the reaction progress curve to the following equation:

$$[product] = v_s t + \left[\frac{v_0 - v_s}{k_{obs}}\right]\left[1 - \exp(-k_{obs} t)\right]$$

Where v_s is the asymptotic velocity and v_0 is the initial velocity. They solved the equation for k_{obs} by iterative nonlinear regression. This equation is empirical not mechanistic.

From a plot of k_{obs} *vs* the initial concentration of the inhibitor they determined k_{on}, the forward rate constant of inhibition (second order), and k_{off}, the dissociation constant for inhibition (first order) by fitting to the linear equation:

$$k_{obs} = k_{on}[I] + k_{off}$$

I am uncertain of the validity of this step. From this analysis they determined the following rate constants for the three boronated proteasome inhibitors:

Proteasome Inhibitor	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_i(nM)	$t_{1/2}$ (min)
PS-341	$3.2x10^{5}$ ($\pm 1.5x10^{4}$)[a]	$5.6x10^{-4}$ ($\pm 1.7x10^{-4}$)	1.8[b] (± 0.52)	20.5 (15.8-28.9)
PS-273	$3.1x10^{5}$ ($\pm 1.6x10^{4}$)	$3.3x10^{-4}$ ($\pm 1.3x10^{-4}$)	1.1 (± 0.44)	35.2 (25.1-57.8)
PS-304	$4.2x10^{5}$ ($\pm 0.9x10^{4}$)	$2.6x10^{-4}$ ($\pm 0.8x10^{-4}$)	0.6 (± 0.19)	44.4 (33.9-64)

a Range of standard deviation.
b. The K_i for PS-341 is reported to be 0.6 nM, however under the assay conditions of this study, a higher value for K_i was obtained.

The authors also determined k_{off} by a second method. They incubated 20S proteasome with purified PA-28 activator protein, substrate and each of the inhibitors at different concentrations. They determined k_{off} as the first order rate constant (single exponential model) of the reappearance of activity.

Proteasome Inhibitor	k_{off} (s^{-1})	$t_{1/2}$ (min)
PS-341	$5.9x10^{-4}$	19.4
PS-273	$3.6x10^{-4}$	31.8
PS-304	N/D[a]	>>1320

a: Could not be determined.

The authors claim that this method is more accurate but it neglects reassociation of the enzyme and the inhibitor, a phenomenon that may not be inconsiderable in this situation. The authors claim that based on this experiment the binding of PS-304 is essentially irreversible, but their inability to determine k_{off} by this method may be due to the contribution of the forward reaction.

I am not sure of the biological significance of these values but they have one clear implication, that is that the forward rate constant for inhibition for this class of inhibitor is much greater than the reverse rate constant. Maintaining even relatively low concentrations of PS-341 (nM) should result in considerable inhibition of the 20S-proteasome proteolytic activity against small peptides. The assay provides no information on the inhibition of the degradation of large ubiquitin-tagged proteins and the physiological significance of the SDS activated reaction remains uncertain.

2) Selectivity and Specificity of PS- 341 (——— Discovery Screen). Module 4. Folder 4211-prim-pd, file panlabs.pdf.

——— a contractor, did this standard battery of *in vitro* pharmacology studies to test for agonist and antagonist activity at numerous pharmacological receptor sites. The study authors do not describe the numerous experimental procedures, nor did they provide references. Thus, I cannot confirm the validity of the tests. Neither does the report provide any interpretation of the studies. I have copied the following tables directly from the report. The results suggest that PS-341 has no significant interactions with any of the tested pharmacological sites at concentrations of ———(the only concentration tested) or less.

1995

[redacted] Data Report

ASSAY			CODE: [redacted] PT#: [redacted] SOLV: 0.5% DMSO MW: 384.25				
			% Inhibition (μM)				
RECEPTOR	LIGAND	SOURCE	10	10	1	0.1	0.01
Angiotensin AT_1	[^{3}H]Losartan	Rabbit Adrenal Gland	-17				
Bradykinin B_2	[^{3}H]Bradykinin	GP Ileum	-18				
$Cholecystokinin_A$	[^{3}H]L-364718	Rat Pancreas	-5				
$Cholecystokinin_B$	[^{3}H]CCK-8	Mouse Brain	-6				
Dopamine D_3	[^{3}H]Spiperone	Human (r)DNA	6				
Dopamine $D_{4.2}$	[^{3}H]Spiperone	Human (r)DNA	7				
Endothelin ET_A	[^{125}I]Endothelin	A10 Cells	6				
Endothelin ET_B	[^{125}I]Endothelin	Rat Cerebellum	-2				
Galanin	[^{125}I]Galanin	Rat Whole Brain	-12				
Histamine H_3	[^{3}H]NAMH	Rat Whole Brain	-19				
Kainate	[^{3}H]Kainate	Rat Whole Brain	-4				
Leukotriene B_4	[^{3}H]LTB_4	GP Spleen	22				
Muscarinic M_1	[^{3}H]Pirenzepine	Rat Brain Cortex	5				
Muscarinic M_2	[^{3}H]NMS	Rat Heart	-1				
Muscarinic M_4	[^{3}H]NMS	Rabbit Lung	-13				
Neurokinin NK_1	[^{3}H]Substance P	GP Submaxil. Gland	1				
Neuropeptide Y_2	[^{3}H]NPY	Rab. Kidney Medulla	-2				
Nicotinic (CNS)	[^{3}H]Cytisine	Rat Brain Cortex	5				
NMDA	[^{3}H]CGS-19755	Rat Brain Cortex	12				
Phencyclidine	[^{3}H]TCP	Rat Brain Cortex	-11				
PAF	[^{3}H]PAF	Rabbit Platelet	15				
Serotonin $5\text{-}HT_{1A}$	[^{3}H]8-OH-DPAT	Rat Brain Cortex	12				
Serotonin $5\text{-}HT_3$	[^{3}H]GR-65630	Rabbit Ileum	-6				
Sigma σ_1	[^{3}H]Pentazocine	GP Brain	-7				
Sodium Channel-2	[^{3}H]Batrachotoxinin	Rat Whole Brain	-14				
Thromboxane A_2	[^{3}H]SQ-29548	Rabbit Platelet	-1				
TRH	[^{3}H](Me)TRH	Rat Whole Brain	-2				
VIP	[^{125}I]VIP	GP Lung	1				
			% Inhibition (μM)				
ENZYME		SOURCE	10	10	1	0.1	0.01
Calcineurin		Rat Brain	2				
Calpain		Human Erythrocytes	-17				
EGF Tyrosine Kinase		Human (r)DNA	-24				
Nitric Oxide Synthase (Constitutive)		Rat Cerebellum	-14				
Nitric Oxide Synthase (Inducible)		RAW 264.7 Cells	-2				
			100	100	10	1	0.1
Protein Kinase Cα		Rat Brain	20				
Protein Kinase Cβ		Rat Brain	24				

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1995

ProfilingScreen Data Report

ASSAY			CODE: [redacted] PT#: [redacted] SOLV: 0.5% DMSO MW: 384.25				
			% Inhibition (μM)				
RECEPTOR	LIGAND	SOURCE	10	10	1	0.1	0.01
Adenosine A_1	[^{3}H]DPCPX	Rat Whole Brain	-2				
Adenosine A_{2A}	[^{3}H]CGS-21680	Rat Striatum	18				
Adrenergic-α_1	[^{3}H]Prazosin	Rat Whole Brain	-12				
Adrenergic-α_2	[^{3}H]Rauwolscine	Rat Whole Brain	10				
Adrenergic-β_1	[^{3}H]CGP-12177	Rabbit Heart	-10				
Adrenergic-β_2	[^{3}H]CGP-12177	Rat Lung	-13				
Calcium Channel (L)	[^{3}H]Nitrendipine	Rat Brain Cortex	-2				
Chloride Channel	[^{3}H]TBOB	Rat Brain Cortex	9				
Dopamine D_1	[^{3}H]SCH-23390	Human (r)DNA	-3				
Dopamine D_{2A}	[^{3}H]Spiperone	Human (r)DNA	8				
Estrogen	[^{3}H]Estrogen	Calf Uterus	-8				
$GABA_A$	[^{3}H]Muscimol	Rat Whole Brain	2				
Glucocorticoid	[^{3}H]Dexamethasone	Human Jurkat Cells	15				
Glutamate	[^{3}H]L-Glutamate	Rat Whole Brain	-3				
Glycine (Strych. Sens.)	[^{3}H]Strychnine	Rat Spinal Cord	-25				
Histamine H_1	[^{3}H]Pyrilamine	GP Brain	-10				
Insulin	[^{125}I]Insulin	Rat Liver	3				
Muscarinic M_2	[^{3}H]NMS	Rat Heart	NS				
Muscarinic M_3	[^{3}H]NMS	Rat Submaxil. Gland	0				
Opiate Delta (δ)	[^{3}H]DPDPE	GP Brain	9				
Opiate Kappa (κ)	[^{3}H]U-69593	GP Brain	-10				
Opiate Mu (μ)	[^{3}H]DAMGO	GP Brain	22				
Phencyclidine	[^{3}H]TCP	Rat Brain Cortex	NS				
Phorbol Ester	[^{3}H]PDBu	Mouse Whole Brain	-13				
Potassium [$I_{K(ATP)}$]	[^{3}H]Glyburide	HIT-T15 Beta Cells	7				
Progesterone	[^{3}H]R5020	Calf Uterus	-12				
Serotonin 5-HT_1	[^{3}H]5-HT	Rat Brain Cortex	7				
Serotonin 5-HT_2	[^{3}H]Ketanserin	Rat Brain Cortex	15				
Sigma	[^{3}H]DTG	GP Brain	-6				
Sodium Channel-2	[^{3}H]Batrachotoxinin	Rat Whole Brain	NS				
Testosterone	[^{3}H]Mibolerone	Rat Prostate	4				

REMARKS:

NS: Not shown because these assays were tested in [redacted] Program.

3) Selectivity of PS-341 for Inhibition of the 20S Proteasome. study 166, Module 4. Folder 4211-prim-pd, file 166.pdf.

Investigators at [redacted] did this study. They used the assay for proteasome activity described above and standard assays for 11 other proteases to determine the inhibition of these

enzyme activities by PS-341. "The panel of proteases was chosen to cover several classes, including serine and cysteine proteases. Critical extracellular and intracellular proteases involved in protein degradation and in activation of blood coagulation were included." All the assays were standardized. The table below shows the apparent inhibitory rate constants $K_{i,app}$ for the 20S proteasome and the 11 other proteases. nM concentrations of PS-341 did not significantly inhibit the activities of these 11 other proteases under standard reaction conditions. It is probably mechanistically significant that the protease with the second lowest K_i was chymotrypsin. It is the chymotrypsin-like activity of the 20S proteasome that is most affected by PS-341.

Enzyme	PS-341^ $K_{i,app}$(nM)
20S Proteasome	0.62
Chymotrypsin	970
Trypsin	>10,000
Cathepsin B	>10,000
Elastase	5,700
Calpain I	>10,000
Calpain II	>10,000
ACE	4,900
Thrombin	>10,000
Coagulation Factor βXIIa	>10,000
TPA	>10,000
Papain	>10,000

^PS-341 was tested at 10μM

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4) The influence of proteasome isoforms on inhibition of proteasome activity by PS-341. RPT- 00064, Module 4. Folder 4211-prim-pd, file rpt-00064.pdf.

Eric Lightcap of Millennium Pharmaceuticals did this experiment to determine whether the kinetics of inhibition of PS-341 varies among different proteasome isoforms and whether different cancer cell lines vary their expression of proteasomes in response to PS-341 inhibition. In these experiments, he referred to the constitutive form of the 20S proteasome as 20Sc and to the γ-interferon induced form as 20Si. He evaluated the following six combinations of these two proteasomes plus the two major activators, 20Sc, 20Si, 19S+ 20Sc, 19S+ 20Si, PA28+ 20Sc, and PA28+ 20Si. Note that in this discussion 19S is the same as the PA700 activator described above. I have maintained Dr. Lightcap's nomenclature to maintain consistency between the review and the report.

The proteasome combinations were prepared by incubating the various purified subunits in buffer for 45 minutes before initiation of the reaction, achieving concentrations of proteasome ranging from [redacted] The proteasome activity was determined by the assay described above using Suc-Leu-Leu-Val-Tyr-AMC, where AMC is adriamycin, as the substrate. The substrate concentration was constant in each assay at 60 μM. Thus, the concentration of substrate was sufficient to achieve saturation and presumably reaction steady state. The reaction determined passive hydrolytic chymotrypsin-like activity. Again, this assay does not determine the ATP requiring protealytic activity of the proteasome toward large ubiquitinated proteins. PS-341 was then added at concentrations ranging from [redacted] to [redacted], concentrations at least 30 times greater than that of the enzyme. The data from the fluorescence curves from each assay was fitted to the following equation:

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Where " y = arbitrary fluorescence units (FU), v_S = steady state velocity (FU/s, velocity after inhibition has equilibrated), v_0 = initial velocity (FU/s, velocity after rapid binding of inhibitor), k_{obs} = rate constant for slow- binding inhibition (min^{-1}), and t = elapsed time (min). The inhibition kinetics for the isoforms represent a single determination. The inhibition kinetics for lysates represent averages of triplicate determinations."

From this imprecise description, it is difficult to determine exactly how the parameters were obtained from the graphical fluorescence data. I assume v_s is the final reaction steady state after inhibition that is measured as the asymptote of the final phase of the reaction curve. In the parameter FU/s – s is undefined in the methods narrative. I am not sure how v_0 was determined. This equation is an observational approximation and not a rigorous kinetic analysis. Nevertheless, applied consistently it probably provides reasonable relative values.

From this, the author determined the steady state velocity of each proteasome preparation; I assume in the absence of inhibitor.

Table 1 Peptidohydrolytic Activity of Proteasome Isoforms

Isoform	Peptide Hydrolysis (FU/s)
20Sc	1.8
20Si	1.8
19S+20Sc	3.9
19S+20Si	9.6
PA28+20Sc	2.5
PA28+20Si	4.0

The table shows that the proteolytic activity of the two 20s isoforms are equivalent in this assay and that the presence of both 19S and PA28 activators enhances that activity as one would expect.

The following table shows the values of the inhibitory constant, k_{obs}, for each of the proteasome preparations in the presence of 195 nM PS-341.

Table 2 Kinetics of Inhibition of Proteasome Isoforms

Isoform	Inhibition Kinetics (kobs, min-1)
20Sc	0.19
20Si	0.40
19S+20Sc	0.31
19S+20Si	0.28
PA28+20Sc	0.24
PA28+20Si	0.23

The author claims that this table demonstrates that the effect of PS-341 is relatively constant across the various preparations. In other analysis, the author demonstrates that k_{obs} varies exponentially with the concentration of PS-341 irrespective of the proteasome isoform. I cannot confirm the validity of this assessment without recourse too more analysis than time permits and without error estimates I cannot comment on the small differences in among the values. Nevertheless, if true the results imply that PS-341 exerts its inhibitory effect at the same binding site and with similar affinity across all these proteasome isoforms. The finding is of academic interest only and has no regulatory implications.

The author also compared the kinetics of inhibition in whole cell lysates (HT-29, HL-60, U266Bi and whole blood) using these same methods. This experiment demonstrated that

inhibition was again similar irrespective of the preparation.

Table 3 **Comparing the Kinetics of Inhibition by PS-341 for Crude Lysates and Proteasome Isoforms**

Proteasome Source	Inhibition Kinetics (k_{obs} min^{-1})
HT-29 lysate	0.17 ± 0.03
HL-60 lysate	0.12 ± 0.02
U266B1 lysate	0.13 ± 0.02
Whole blood lysate	0.146 ± 0.003
20Sc	0.12
20Si	0.10
19S+20Sc	0.12
PA28-20Sc	0.08

Using gel electrophoresis the author demonstrated that the distribution of proteasome isoforms in U266Bi, RPMI- 8226, and MM1 cell lines was unchanged when the cells were treated with vehicle, 5, 25, and 50 nM PS- 341. These experiments are not rigorous because they do not account for differential transfer of the proteins to the gel and the gels are not well resolved.

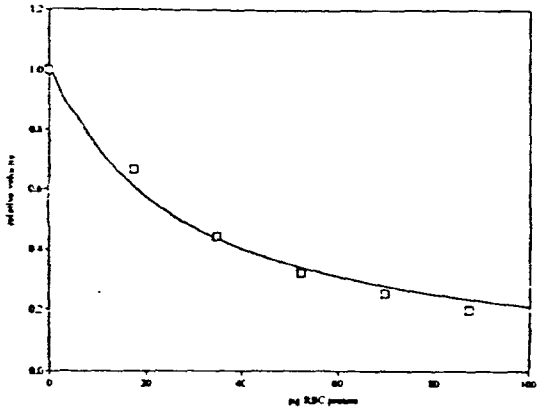
These experiments suggest that the mechanism of inhibition of PS-341 is similar irrespective of the proteasome isoform. It also implies that the binding is similar across isoforms and that treatment with PS-341 does not alter the distribution of proteasome isoforms across several tested cell lines. Nevertheless, none of these analyses are rigorous and the experimental documentation lacks considerable detail. These experiments are inadequate for regulatory purposes.

5) Accurate measurement of proteasome inhibition: Novel ex vivo *assays. Study 167, Module 4. Folder 4211-prim-pd, file 167.pdf.*

Researchers at ———— completed these experiments in October of 1999. They did these experiments in order to discover optimal conditions for the standard assay (above in study #1, and #7 below) of the chymotryptic activity of the 20S-proteasome when they applied the assay to *ex vivo* blood samples. They also wanted to establish a way to determine the extent of proteasome inhibition in the blood of patients and animals treated with PS-341 absent an untreated control sample.

Evidently, Stein *et al.*[4] did not develop the original assay using blood or white cells, the tissue of interest in any clinical study of multiple myeloma. The graph below shows that proteins extracted from lysed RBCs inhibits proteasome activity in the standard assay with an inhibitory constant of about 13 µg/ml. Lysed RBC proteins did not inhibit the fluorescence of dissolved ————, the flurophor hydrolyzed from Suc-Leu-Leu-Val-Tyr-AMC in the standard assay, thus eliminating quenching as the source of the inhibition.

Figure 1.



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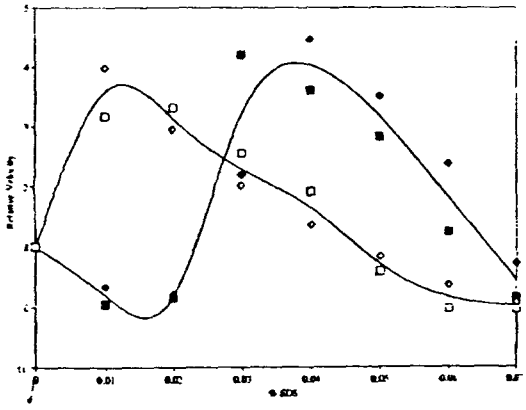
The investigators were not able to identify the components of the RBC lysate that caused this inhibition. Other experiments established that the inhibition was not substantially due to hemin, globin, bilirubin, biliverdin, hemoglobin, or methemoglobin. The RBC lysate did not inhibit proteasome tryptic activity. This assay is similar to the chymotryptic assay but the substrate is 10 pM Bz-Val-Gly-Arg-AMC in 0.035% SDS. The actual cause the RBC lysate inhibition remains unknown.

The first change in the standard assay that resulted in improved results was to increase the concentration of Suc-Leu-Leu-Val-Tyr-AMC, the chymotryptic substrate, from ———— To increase substrate solubility and stabilize the solution they increased the concentration of DMSO to 1%. Next, the investigators optimized the concentration of SDS. The following graph shows that the reaction that for purified proteasomes (open squares and diamonds) the optimum is about 0.01% and that for WBC (closed squares and diamonds) lysate it is 0.03 to 0.04%. The investigators decided to use 0.035% in subsequent assays. They determined that carbon chain length of the SDS did not affect the assay.

Figure 2.



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In the presence of 0.035% SDS the chymotryptic activity of RBC lysate increased hyperbolically toward an asymptotic maximum. The reaction was linear with added lysate protein only with amounts of about 25 µg or less. But, the reaction became linear with added lysate protein when the investigators increased the SDS concentration to 0.05% as the following graph shows.

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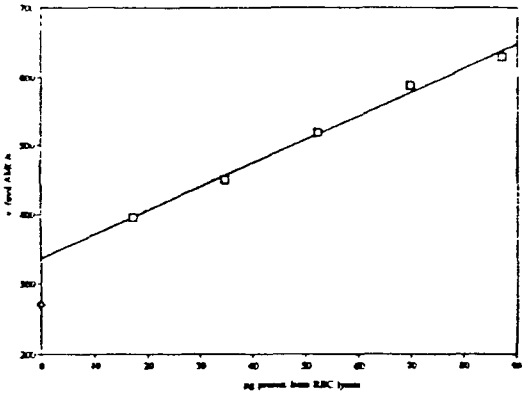
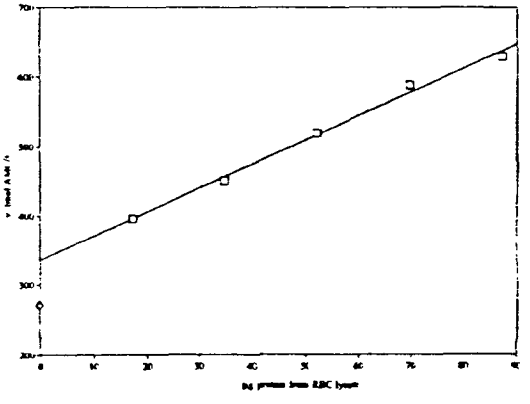
Figure 3.



More importantly, when the investigators determined WBC chymotryptic activity under these conditions and in the presence of added RBC lysate the reaction remained linear. This is the same experiment as shown in figure 1 above but with a strikingly different result.

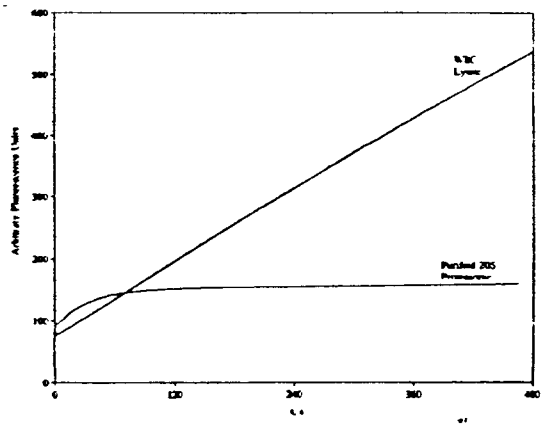
Figure 4.



Thus, the chymotryptic activity of WBC lysate, free of RBC contamination, was optimal using the 0.035% SDS buffer. The activity of RBCs or packed whole blood (PWB, includes RBC, WBC, and platelets) lysate was best measured with the 0.05% SDS buffer.

The following graph shows the time course of the chymotryptic proteolysis using WBC lysate and purified proteasome in the presence of 0.05% SDS. The WBC lysate reaction proceeds linearly for at least 400 seconds, but the activity of purified proteasomes is hyperbolic with a linear phase lasting well less than 30 seconds. This is consistent with the SDS optimization experiment described above. This result suggests that the activity measured in the WBC and RBC lysate experiments may not be due to the 20S proteasome but to some other protease.

Figure 5.

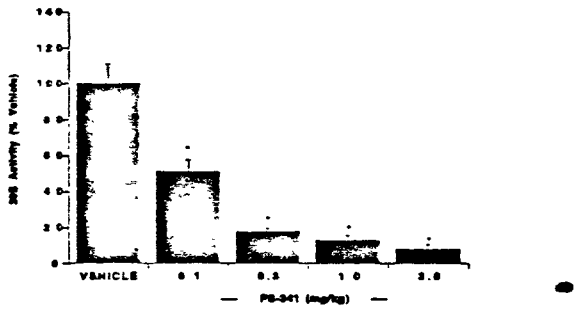


In subsequent experiments the investigators attempted to define a normalization procedure based on the tryptic activity of the lysates as an alternative to normalization against the protein concentration. This and other parts of the assay depend on the assumption that the 20S-proteasome is responsible for all or most of the chymotryptic activity. Without considerably more rigorous kinetic analysis and more time, I cannot confirm the validity of this method of determination of 20S-proteasome activity in *ex vivo* samples. The development of these assays is an attempt to determine the pharmacodynamics of PS-341.

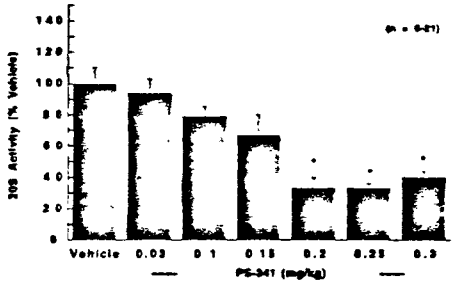
6) Temporal 20S proteasome activity in isolated white blood cells and tissues from mice or rats after intravenous dosing with PS-341. Module 4. Folder 4211-prim-pd, file pcol-06-01.pdf.

Researchers at [redacted] completed these experiments in May of 1998. The purpose of these experiments was to determine the time course of the inhibition of proteasome activity by PS-341 *in vivo* in rats and mice. The investigators used female CD2-Fl mice (18 to 20 g), female BALB/c mice (18 to 20 g), female Wistar rats (150 to 200 g) and male Sprague-Dawley rats (250 to 450 g). They gave PS-341 doses intravenously using a dose volume of 100 µL per mouse or 1.0 mL/kg in rats. Control groups were given vehicle (98% saline [0.9%], 2% ethanol, 0.1% ascorbic acid). They collected tissues at various time points and determined the protealytic activity using the methods described above.

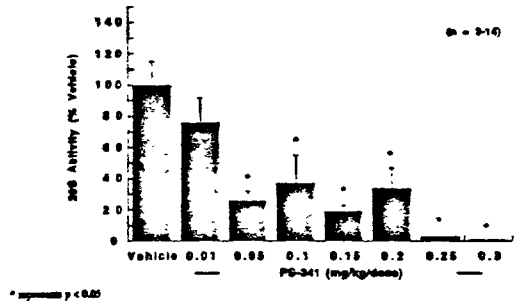
The following graph shows that activity in mouse WBCs determined one hour after a single dose of PS-341 decreased with increasing dose. In this experiment, five mice were used for each dose point and the samples were pooled to obtain the result. A similar experiment in rats showed a very similar dose related decrease in activity after a single dose.



In mice, these single doses above are 0.3, 0.9, 3 and 9 mg/m^2. The activity in isolated mouse WBCs 24 hours showed no clear dose relationship but there was significant recovery in all dose groups. The following graph shows that in rats the recovery of activity 24 hours after a single dose was dose dependent. The doses in this experiment were 0.18, 0.6, 0.9, 1.2, 1.5 and 1.8 mg/m^2. Clinically humans tolerate 1.2 and 1.5 mg/m^2. Activity returned to near control levels by 48 hours after dosing.



In multiple dose studies, inhibition was again dose-dependent in rat WBCs, but the experiment showed considerable variability. The following figure shows 20S-proteasome activity in isolated rat WBCs 24 h after daily IV dosing with PS-341 for seven consecutive days.



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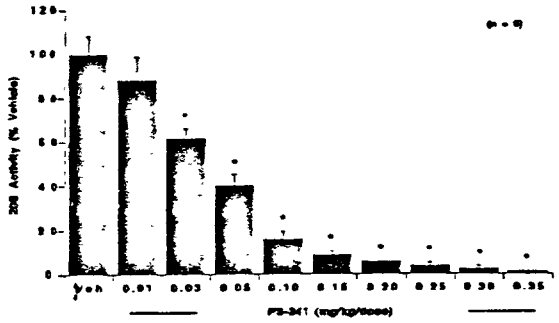
At the highest dose, the activity 24 hours after seven doses is markedly less than that at 24 hours after a single dose. This could represent drug accumulation or a drug related decrease in proteasome expression. The pharmacokinetics of PS-341 (see section by Dr. Goheer) and the tight binding demonstrated in the enzyme kinetics experiments above suggest that the former is most likely. Daily doses of PS-341 over any extended period are poorly tolerated across species; the tolerated dose goes down significantly when compared to more protracted dose schedules. This graph probably explains that observation. It also suggests that PS-341 binding is not rapidly reversible *in vivo* consistent with the *in vitro* kinetics.

The following figure shows the dose dependent decrease in proteasome activity in rat WBCs one hour after the last of four doses given twice weekly for two weeks. This is the schedule now used most frequently clinically and the one proposed for registration. As the kinetics predicts, inhibition increases exponentially with dose and is nearly as great as that seen with seven daily doses.



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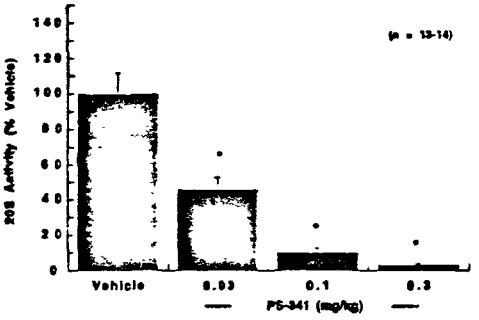
The following graph shows that inhibition in the liver is dose dependent and rapid. Here a single dose was given to rats and the liver was assayed for proteasome activity one hour later.

Figure 10. 20S Proteasome Activity in Isolated Rat Liver 1.0 h after a single IV Dose of PS-341



* represents $p < 0.05$

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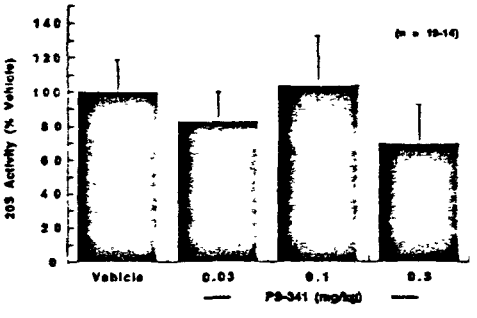
In the liver, the percent inhibition after a single dose appears to be less than five percent after a single dose in rats, whereas in rat WBCs the inhibition was only about 18% one hour after a single dose (not shown). This implies that liver is more affected than WBCs and consequently some difference between the proteasome activities of these cells. It also suggests that WBCs may not be a good surrogate for PS-341 pharmacodynamics. At 24 hours, activity in the liver had recovered to about 35% of control values in the high dose (0.3 mg/kg) group and recovery was almost complete at 48 hours.

A single dose of PS-341 did not inhibit proteasome activity in rat brain to the extent that it did in liver or WBCs. The graph below shows only about 60% inhibition in the high dose group. The authors of this study postulate this is due to decreased cellular uptake.

Figure 13 20S Proteasome Activity in Isolated Rat Brain 1.0 h after a single IV Dose of PS-341



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A single dose of PS-341 also caused a dose dependent decrease in proteasome activity in other tissues measured one hour after dosing in rats. The highest single dose of PS-341, 0.3 mg/kg caused a significant decrease in 20S proteasome activity in colon (~20% of control activity), gastrocnemius muscle (~10%) and the prostate (~30%). PS-341 did not inhibit proteasome activity in rat testes.

Thus, inhibition of proteasome activity increases exponentially with increasing dose when measured *ex vivo* after single and multiple doses. Activity recovers more quickly in the mouse

than in the rat. In both species, recovery is essentially complete after 48 hours. Based on the time course, recovery probably occurs via detachment of PS-341 from the inhibitory site and not by *de novo* synthesis of new proteasome. Activity recovers significantly less quickly after multiple doses in the rat and inhibition is more complete at a given dose. Inhibition is more profound in the liver than in WBCs at a given dose. Inhibition occurs in other tissues such as colon, muscle and prostate but to a lesser extent than in liver. PS-341 does not inhibit proteasome activity in brain or testes consistent with pharmacokinetic studies of the distribution of radiolabeled drug.

7) Development and validation of the 20S proteasome assay for detecting residual 20S activity in rat peripheral blood leukocytes: in vivo *regulation of proteasome activity in rats by the boronate proteasome inhibitor PS- 341. Study 159, Module 4. Folder 421-prim-pd, file 159.pdf.*

Investigators at [redacted] completed these experiments in July of 1997. They designed these studies to establish the conditions for an assay to detect 20S-proteasome proteolytic hydrolysis activity in rat peripheral-blood-leukocytes.

The investigators determined 20S-proteasome activity by measuring the rate of proteolytic hydrolysis of a [redacted] tagged peptide substrate by the cell lysate sample and normalizing the activity to the amount of cell-specific protein present in the lysate. In the assay, [redacted]

The measurement of intra-assay variation of the 20S assay in rat PBMNC had a coefficient of variation (CV) of less than 10%. The inter-assay variation showed a CV of less than 15%.

The authors then demonstrated that PS-341 inhibited the 20S-proteasome activity *ex vivo* in rat peripheral blood leukocytes following a single IV dose. The minimal effective dose (MED) was about 0.1 mg/kg when they measured activity one hour after dosing, and 0.2 mg/kg when measured 24 h after treatment. Seven daily IV doses of PS-341 or doses given every other day over a 14 day period causes inhibition of 20S activity at lower doses (MED = 0.05-0.01 mg/kg and <0.1 mg/kg, respectively). The following graphs are examples of these results.

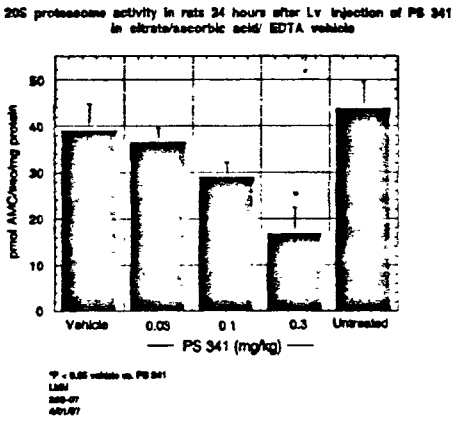

Figure 8 Groups of 5 female Lewis rats (150-180 g) were injected iv via the tail vein with either vehicle (citrate/ascorbic acid/ethanol) or varying doses of PS341 (0.3, 0.1 and 0.03 mg/kg). Naive animals were also used as untreated controls. After 24 h, the animals were anesthetized with ketamine/xylazine and bled through open cardiac puncture. The heparinized blood (2 ml) was processed as described and assayed for 20S activity. Data are expressed as the mean specific activity ± standard error

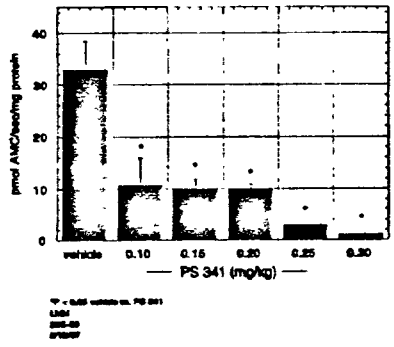

Figure 10 Groups of 6 female Lewis rats (150-180 g) were injected iv once a day via the tail vein with either vehicle (citrate/ascorbic acid/ethanol) or varying doses of PS-341 (0.3, 0.25, 0.2, 0.15 or 0.1 mg/kg) for 7 consecutive days. Animals from the 2 highest dosage groups (0.30 and 0.25 mg/kg) displayed overt signs of toxicity including lethargy, piloerection, diarrhea, yellow serum, enlarged livers and death (4/6 dead at 0.3 mg/kg). Animals were allowed to rest 24 h after the 7th dose and then bled through open cardiac puncture. The heparinized blood (2 ml) was then processed as described and 20S assays performed. Data are expressed as the mean specific activity ± standard error. Some groups contain less than 6 data points because sample parameters and requirements were not satisfied.

These experiments do not establish conclusively that the proteolytic activity observed with this assay is solely attributable to the 20S-proteasome. Neither do they conclusively

establish the validity of the protein normalization procedure. Thus residual activity cannot yet be used as a reliable surrogate for the pharmacodynamics of PS-341.

8) 20S proteasome activity levels in peripheral white blood cells of human volunteers. Study 158, Module 4. Folder 4211-prim-pd, file 158.pdf.

Investigators at [redacted] completed these experiments in May of 1998. They designed these studies to determine 20S-proteasome chymotryptic activity in peripheral white blood cell samples from human volunteers. The methods are similar to those described above in study #7. The investigators collected five blood samples from five volunteers over a period of ten weeks. They determined that the average 20S-proteasome activity in this population was 12.12±0.81 pmol [redacted]'sec/mg protein with individual observed values ranging from 53% to 165% of this value. Across duplicate test samples through the time range of the study, the average variation was 10.0%. The range for individual test duplicates from 0.8% to 22.9%.

9) Assay for Potential Drug Resistance Mechanisms of PS- 341. Study rpt-00047. Module 4. Folder 4211-prim-pd, file rpt-00047.pdf.

Investigators at Millenium Pharmaceuticals completed these experiments in October of 2002. They designed these studies to determine potential mechanisms of drug resistance to PS-341 in mammalian cells.

10) Effect of PS- 341 on Paraprotein Levels in Multiple Myeloma Cells In Vitro.
Study rpt-00043. Module 4. Folder 4211-prim-pd, file rpt-00043.pdf.

Investigators at Millennium Pharmaceuticals completed this study in October, 2002. They designed these studies to determine whether treatment with PS- 341 decreased the expression of paraprotein in multiple myeloma cells *in vitro*. Paraprotein is an immunoglobulin secreted by malignant multiple myeloma cells. According to the study investigators, high concentrations of paraprotein in the blood of multiple myeloma patients correlate with a poor prognosis.

of PS-341 *in vitro*. They identified the proteins by Western blot. They found that 1 and 10 nM PS- 341 inhibites the 26S proteasome and induces the expression of markers of apoptosis in the cells (c-jun and β-catenin). Nevertheless, treatment with PS- 341 did not significantly effect paraprotein concentrations. According to the introduction of this study, patients treated with PS-341 have a decrease in paraprotein concentrations in the blood. This is perhaps somewhat counter-intuitive. One might expect paraprotein concentrations to increase if the proteasome is responsible for its degradation. These *in vitro* experiments suggest that the reduction in paraprotein seen clinically after PS-341 treatment results from a decrease in the number of multiple myeloma cells. This is further evidence that PS-341 might have cytotoxic mechanisms other than inhibition of the 20S-proteasome.

11) Effect of the Proteasome Inhibitor PS-341 in the Human Colon HT-29 Cancer Model. Study pcol-0201, Module 4. Folder 4211-prim-pd, file pcol-02-01.pdf.

Investigators at [redacted] completed these experiments in May of 1998. They implanted human HT-29 cells (5X106) subcutaneously in the hind leg of female nude mice (no further description). They allowed the tumor implants to grow for either 4 or 18 days than began daily IP dosing with PS-341 Monday through Friday (five doses per week) until day 45. Thus one group of mice got 30 injections and one group got 20 injections. The following table shows that PS-341 delayed the growth of tumor implants. Delay increased with increasing dose and with the increased number of doses.

Effect of PS-341 on tumor volume of mice implanted with HT-29 colon tumor cell

Treatment/Regimen	Days To Tumor Volume Of 500 mm³	1000 mm³	Days Of Tumor Growth Delay To 500 mm³	1000 mm³
Dosing Days 4 - 45 (30 doses)				
Untreated	24.8 ± 4.6	31.8 ± 3.2	—	—
Vehicle	24.2 ± 1.9	33.0 ± 3.0	-0.6	1.2
PS-341 (0.03 mg/kg)	23.8 ± 0.8	32.7 ± 1.3	-1.0	0.9
PS-341 (0.1 mg/kg)	26.8 ± 3.4	36.1 ± 3.0	2.0	4.3
PS-341 (0.3 mg/kg)	31.8 ± 4.8	40.3 ± 4.0	7.0	8.5
Dosing Days 18 - 45 (20 doses)				
Untreated	24.8 ± 4.6	31.8 ± 3.2	—	—
PS-341 (0.03 mg/kg)	24.4 ± 1.6	31.6 ± 2.2	-0.4	-0.2
PS-341 (0.1 mg/kg)	23.0 ± 3.3	33.7 ± 3.3	-1.8	1.9
PS-341 (0.3 mg/kg)	25.7 ± 4.0	35.6 ±4.2	0.9	3.8

Data are presented as mean ± sem n = 10 except untreated group where n = 9.

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The investigators say that numerous mice died or became moribund during the study but provide no detail. The dose intensity in this experiment is unusually high. The deaths were probably due to PS-341 treatment. Because of the deficiencies in the reporting, this study is not very useful.

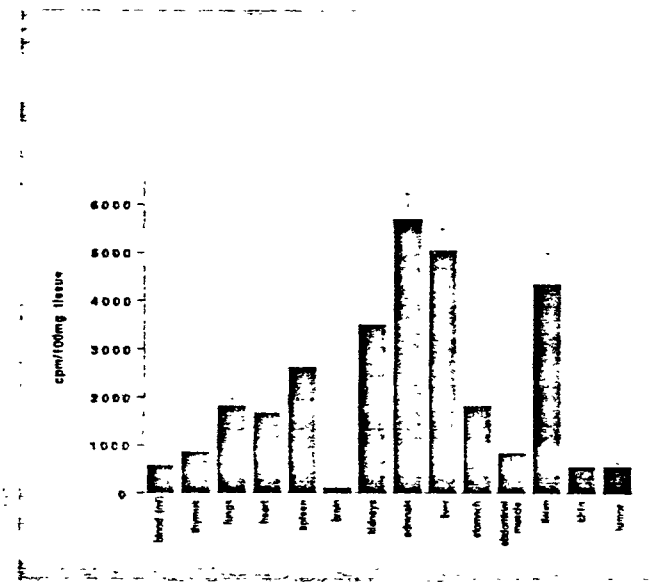
13) Effect of the inhibitor PS- 341 in the Lewis lung carcinoma mouse model. Not Reviewed.

14) Uptake of radiolabeled ^{14}C- PS- 341 into B-16 tumor bearing mice. Module 4. Folder 4211-prim-pd, file pcol-07-01.pdf.

Researchers at ———————————, completed these experiments in May of 1998. The purpose of the study was to determine the distribution of PS-341 in solid implanted tumor relative to other organs. The researchers implanted B16 melanoma cells subcutaneously in female C57BL/6 mice. The tumors were allowed to grow for 14 days, then they gave the mice a single IV dose of ^{14}C-PS-341 (100 pCi/kg; 0.3 mg/kg, the highest dose given in studies that showed marked inhibition in WBCs and liver). They harvested tissues one hour later and analyzed for the tissue concentration of PS-341 by ————

The following graph shows that the amount of radioactivity in the skin and the subcutaneously implanted B16 tumors was low compared to other organs including the adrenals, ileum, liver, kidneys and the spleen. The study is poorly documented; the graph is the only useful data.

Figure 1 Uptake of ^{14}C-PS-341 into tissues 1.0 h post-dose in B16-tumor bearing mice



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The results suggest that PS-341 does not distribute readily to solid tumor implants, but that it does distribute well to highly perfused organs such as kidney, liver and adrenals. I can draw few conclusions about the relevance of these results to the human clinical situation. Nevertheless, implanted melanoma is usually highly vascular. This would imply a deficiency in drug uptake rather than a lack of perfusion. These results are most inconsistent with the sponsor's assertion that PS-341 has greater activity against tumor cells than against normal cells.

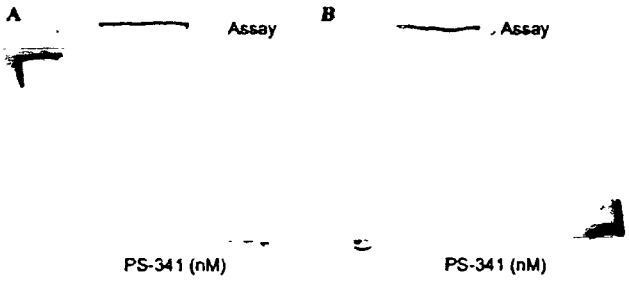
15) RPT- 00070: Development and Characterization of PS- 341- Resistant Multiple Myeloma Cell Lines.
Study RPT-00070, Module 4. Folder 4211-prim-pd, file rpt-00070.pdf.

Investigators at Millennium Pharmaceuticals completed this experiment in October of 2002. The goal of this work was to establish an *in vitro* model of cell resistance to PS-341 and to examine the properties of that resistance. The investigators chose two multiple myeloma cell lines [redacted] They cultured both cell lines independently in the presence of increasing concentrations of PS- 341 for 5.4 months. They then assessed the viability, proliferation, and survival of cell populations and clonal isolates. They also determined the functionality of the proteasome and cross-resistance to other inhibitors of the proteasome and to standard chemotherapeutic agents for both the [redacted] cells. They tested PS- 341- [redacted] cell lines in a mouse xenograft model to evaluate the relevance of resistance *in vivo*.

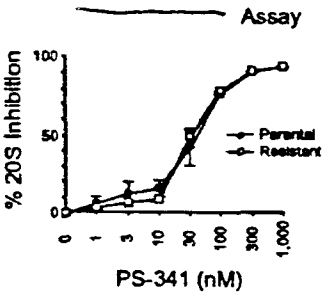
I. [redacted]

The following graph shows the difference in cell viability between parental line [redacted] and resistant line [redacted] of the [redacted] cells (clones selected at random) using the [redacted] assay and a [redacted] assay. The LD_{50} among the various clonal lines increased from between 10 to 50 nM to between 5 to 25 μM (100 to 2500 fold). Other assays for viability showed similar results.

Figure 1 Characterization of PS-341-resistant [redacted] Comparison of [redacted]



Using the [redacted] assay described above (study 1 and others), the investigators determined that the IC_{50} for inhibition of the standard proteolysis reaction was about 30 nM for lysed cells in culture (both [redacted]



In a Western blot analysis to examine accumulation of short-lived proteasome substrates in ——— cells over time after exposure to PS-341, the investigators found a similar pattern of accumulation of ubiquitinylated proteins. They also found similar intracellular accumulation of the ubiquitin proteasome substrates p21, p27, p53, c-myc, c-jun, cyclin B1 and β-catenin. Thus, the mechanism of resistance did not affect the ability of PS-341 to inhibit the proteasome.

As mentioned above, several natural product inhibitors of the 20S-proteasome have been identified. The following table shows that the resistance developed in the ——— cell line did not impart resistance to any of the other tested proteasome inhibitors. The table also shows that PS-341 resistance did not confer resistance to cis-platinum.

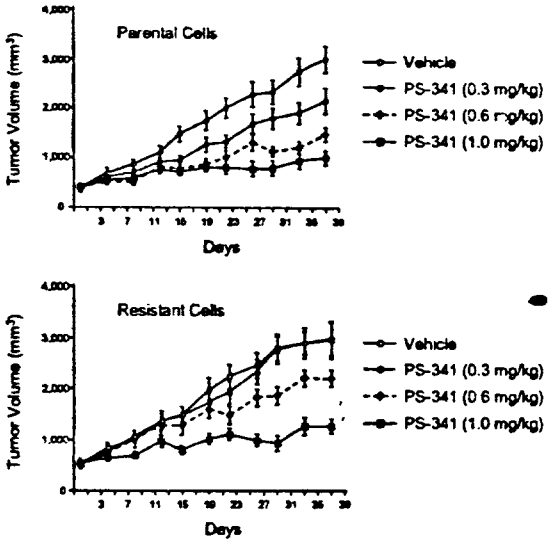
Table 1 **LD_{50} (μM) Values Describing the Effects of Proteasome Inhibitors and Cisplatinum on Viability of ———**

	LD_{50} (μM) Parental	LD_{50} (μM) Resistant
Proteasome Inhibitors		
PS-341	0.03	50
Lactacystin	1	1
MG-132	4	4
PSI	2	8
Epoxomicin	0.1	0.12
Chemotherapeutic Drug		
Cisplatinum	0.12	0.13

Note: The MTT cell viability assays were performed 48 hours after addition of the indicated compounds to the cells.

Nevertheless, when these investigators implanted both the parental and the resistant cell clones into balb/C mice the tumors grew at the same rate while the mice were dosed with PS-341.

Figure 3



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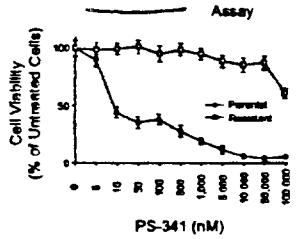
One would have expected the resistant cells to grow more rapidly than the parental in the presence of PS-341.

II.

The following graph shows the difference in resistance to PS-341 between parental and resistant cells after five months of selection. The LD_{50} for parental cells is about 10 nM and that for resistant cells is about 100 uM, or about a 10,000-fold difference.

Figure 5 Characterization of PS-341-resistant



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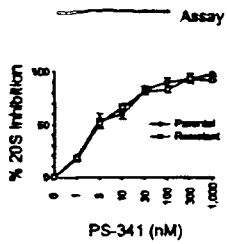
But again, proteasome inhibition in these cells was unchanged by the selection process.

Figure 6 Proteasome Inhibition by PS-341 is Equivalent in



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In [redacted] cells, resistance did confer resistance to the toxicity of the other proteasome inhibitors and to cis-platinum as the following table shows. This result implies that the mechanisms of resistance that evolved in the [redacted] clone involved more radical changes in the proteasome than those that led to resistance in the [redacted] clonal line described above.

Table 2 LD_{50} (μM) Values Describing the Effects of Proteasome Inhibitors and Cisplatinum on Viability of Parental [redacted]

	LD_{50} (μM) Parental	LD_{50} (μM) Resistant*
Proteasome Inhibitors		
PS-341	0.001	>100
Lactacystin	2	>100
PSI	32	>100
Epoxomicin	0.05	>100
Chemotherapeutic Drug		
Cisplatinum	10	>100

The investigators used a Western Blot to demonstrate that [redacted] resistance was not due to overexpression of an [redacted]

This series of experiments shows that cell lines can become resistant to PS-341 cytotoxicity *in vitro*, but in both cases, this resistance did not alter proteasome inhibition by PS-341 in cell lysate determined by the standard [redacted] assay. This implies resistance was not due to an alteration of the chymotryptic active site. In [redacted] cells, the resistance did not convey cross-resistance to several other established proteasome inhibitors. This implies a difference in the mechanism of inhibition between PS-341 and the natural product inhibitors in these cells. These other inhibitors probably act to prevent substrate access to the interior catalytic site of the proteasome so one might not expect cross-resistance between them and PS-341 in all cases. But, resistance in [redacted] did convey resistance to the same set of proteasome inhibitors suggesting an alteration in the structure of the opening of the proteasome. All these results suggest that the cytotoxicity of PS-341 *in vitro* may involve mechanisms other than simple inhibition of the 20S proteasome. This argues against any claims of active site specificity.

The development of resistance did not alter the expression of efflux pumps in resistant [redacted] Neither did the development of *in vitro* resistance alter the response of tumor implants to PS-341 *in vivo*. This result also suggests the possibility of toxic mechanisms associated with PS-341 other than proteasome inhibition.

16) Effects of PS-341 on Hematopoietic Stem Cell Function in a Murine Bone Marrow Transplant Model.
Study RPT-00106, Module 4. Folder 4211-prim-pd, file rpt-00106.pdf.

Investigators at Millennium Pharmaceuticals did this study in October of 2002. They designed these experiments to evaluate the effect of PS-341 on hematopoietic stem cell function. They treated mice with saline or PS-341at 0.6 or 0.8 mg/kg on days 1, 4, 8, and 11 of a 21-day

dosing cycle, for four cycles (16 doses). They then harvested bone marrow cells. They then used the cells for hematopoietic progenitor assays including colony forming unit-granulocyte macrophage (CFU-GM), colony forming unit-erythroid (CFU-E), and high proliferative potential-colony forming cell (HPP-CFC) assays. They also transplanted the cells into lethally-irradiated mice.

They observed no differences in the number of hematopoietic bone marrow progenitor cells from saline or PS-341-treated animals in the *in vitro* assays. Cells from PS-341 treated mice engrafted in the radiated mice as well as cells from controls. When they compared the peripheral blood cell counts of animals transplanted with marrow from either saline treated or PS-341 they observed no difference in the recovery of platelets, white blood cells (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils red blood cells, hemoglobin, hematocrit or reticulocytes. Thus, PS341 treatment had no effect on hematopoietic stem cell function, bone marrow progenitors, and engraftment in lethally irradiated untreated mice.

17) 20S Proteasome Activity Analysis of PS- 341 Drug Substance Impurities. Study rpt-00126, Module 4. Folder 4211-prim-pd, file rpt-00126.pdf.

Investigators at Millennium Pharmaceuticals did this study in October of 2002. They designed these studies to determine whether impurities in the PS-341 drug substance could inhibit 20S-proteasome activity. They identified PS-341-related impurities that existed in the drug substance at concentrations of 0.1% or greater. They synthesized and analyzed these to determine their ability to inhibit 20S-proteasome activity. They determined the second order rate constant (k_{obs}/[I] where I is the inhibitor) as an approximation of binding affinity. All of the non-boronated impurities and all but two of the boronated impurities were relatively inactive. Initial analysis showed that impurity F and J had k_{obs}/[I] values three to four fold higher than PS-341 suggesting that they were more active. Both of these impurities are tripeptide boronates. The investigators then analyzed the kinetics of these impurities in more detail using the methods described above (study 1). They compared the kinetics of these impurities to the drug substance and to highly purified PS-341. The results of this analysis listed in the table below showed that the forward rate constants for these two impurities are greater than that of the drug substance or purified PS-341. Substantial concentrations of these impurities could increase the ability of the drug substance to inhibit 20S-proteasome activity. The experiment provides no information on the relative toxicity of these impurities, but based on these results one would expect them to be somewhat more toxic than PS-341. Nevertheless, at low concentrations (1% or less), these impurities are probably inconsequential. The report does not state typical concentrations for these impurities. According to the review chemist the concentrations of these impurities is less than 1%.

Table 3 Comparison of Inhibition Constants for Ultra-pure PS-341, PS-341 Drug Substance, Impurity F, and Impurity J

	K_i (nM)	k_{on} ($nM^{-1}s^{-1}$)	k_{off} (s^{-1})
Impurity F	[redacted]		
Impurity J	[redacted]		
PS-341 Drug substance	0.69	$(2.61 \pm 0.15) \times 10^{-4}$	$(1.81 \pm 0.67) \times 10^{-4}$
PS-341 Ultra-pure	0.74	$(2.65 \pm 0.16) \times 10^{-4}$	$(1.95 \pm 0.91) \times 10^{-4}$

k_{on}, k_{off}, and K_i were calculated as described in Section 5.2.

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18) Relative Expression of Selected Genes in the Ubiquitin Proteasome Pathway in Normal and Cancerous Tissue. Study 00044, Module 4. Folder 4211-prim-pd, file rpt-00044.pdf.

Investigators at Millennium Pharmaceuticals did this study in October of 2002. They designed this study determine whether ubiquitin-proteasome pathway genes are elevated in human solid tumors when compared to corresponding benign tissues. They measured the expression of a representative set of ubiquitin-proteasome pathway genes in human solid tumors and corresponding normal tissue by quantifying mRNA from these genes. The experiments show that expression of genes encoding for proteins of the ubiquitin proteasome pathway were elevated in colon and lung primary tumors and in metastatic colon carcinoma to the liver when compared to corresponding normal tissue. Increased expression in primary breast and ovarian malignancies was less pronounced. The following table provides an example of these results. The proteins listed are proteasome subunits. These experiments did not involve PS-341 treatment.

Table 1 Proteasome Subunit Expression in Tumor and Normal Human Tissue

Tissue Type	PSMA5	PSMA6	PSMB5	PSMD14
Breast Normal	82.3	2.4	51.3	19.9
Breast Tumor	159.4	3.0	230.8	44.8
(Fold Change)	1.9	1.2	4.5	2.3
Ovary Normal	126.2	1.5	75.2	6.4
Ovary Tumor	112.9	16.7	53.3	19.5
(Fold Change)	0.9	11.3	0.7	3.0
Lung Normal	26.3	0.4	15.9	3.2
Lung Tumor	117.4	9.6	59.2	30.3
(Fold Change)	4.5	25.8	3.7	9.5
Colon Normal	71.8	0.3	17.7	6.1
Colon Tumor	349.3	35.9	81.0	61.8
(Fold Change)	4.9	132.8	4.6	10.2

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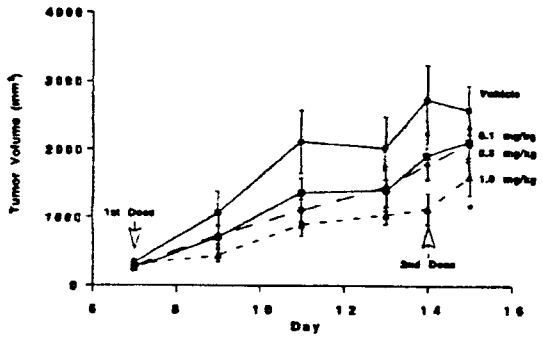
12) Effect of the inhibitor PS- 341 in the Lewis lung carcinoma mouse model. Study 05-01. Module 4. Folder 4211-prim-pd, file pcol-05-01.pdf.

Investigators at [redacted] completed these experiments in May of 1998. The experiments were designed to show potential efficacy against Lewis lung carcinoma tumor implants *in vivo*.

The investigators injected $2X10^6$ Lewis lung carcinoma cells subcutaneously in the hind legs of male C57BL/6 mice. The tumors reach 100 mm^2, at approximately day 7. They then dosed the animals intravenously using a dose volume of 100 µL per mouse. Control groups were dosed with the vehicle (98% saline [0.9%], 2% ethanol, 0.1% ascorbic acid). In the first experiment, they dosed the animals (10 per group) on day 7 and day 14 (weekly regimen). The following graph shows that PS-341 slowed tumor growth dependent on the dose.



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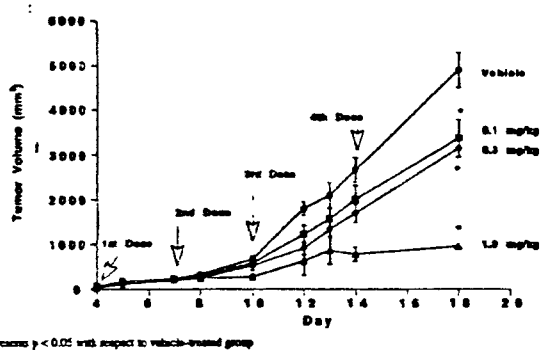
In the second experiment they dosed the mice twice weekly for two weeks. The following experiment shows that this regimen was more effective at delaying tumor growth at a given dose. This schedule is the one the FDA is currently considering for registration.



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Pharmacology Summary

PS-341 (Velcade) reversibly inhibits the chymotryptic proteolytic activity of the 20S-proteasome of mammalian cells. The 20S-proteasome binds with several regulatory proteins to create the 26S-proteasome complexes that hydrolyzes proteins that have been marked for destruction by the ubiquitin enzyme cascade. This ubiquitin-proteasome system is responsible for essential elements of homeostatic control within the cell in G_0 and numerous processes through the course of the cell cycle. When PS-341 inhibits this system, the cell cycle arrests at the transition G_2-M. Inhibited cells then initiate apoptosis. Two [redacted] impurities of the drug product are two to three times more active than PS-341 itself, but their concentration in the drug product is less than 1%.

The equilibrium constant for the binding of PS-341 to the active site of the 20S-proteasome is about 2 nM; the reverse reaction is slow. Inhibition of the chymotryptic proteolytic activity is about the same irrespective of proteasome isoform. In a standard screen, PS-341 at a concentration of 10-μM did not significantly interact at a variety of well-characterized pharmacological receptors *in vitro*. This does not discount the possibility of interactions at yet uncharacterized cellular sites. Neither did PS-341 significantly inhibit a variety of other proteases at μM concentrations.

When PS-341 is given to animals or to humans, the inhibition of the chymotryptic proteolytic activity can be measured *ex vivo* in the lysate of isolated WBCs. Contamination by RBC lysate interferes with this measurement and the assay is difficult to normalize for proteasome content. Activity measured in WBCs from treated animals or humans recovers to normal in about 48 hours suggesting elimination of the PS-341 as opposed to [redacted] synthesis of proteasome. Repeat dosing causes significantly greater inhibition compared to a single dose at the same level (about 30% after a single dose compared to almost 99% after seven doses in WBCs *ex vivo*). Inhibition could be detected in tissue from colon, muscle, prostate and liver but little was seen in the testes or brain of rodents. The inhibition in the liver was significantly greater than in WBCs.

Cells can develop resistance to PS-341 cytotoxicity over time *in vitro*. This resistance is probably not mediated by over-expression of transmembrane molecular pumps such as MDR. In some cases, the acquired resistance can confer resistance to other proteasome inhibitors. The ubiquitin-proteasome system is probably responsible for the degradation of cytochrome P450 isoenzymes. Thus, in concert with induction, degradation by the proteasome may be essential to the maintenance of appropriate intercellular concentrations of cytochrome P450. In the presence of PS-341 some cytochrome P450 concentrations may increase resulting in altered metabolic response to other drugs or chemicals.

PS-341 treatment does not affect the expression of paraprotein in multiple myeloma cells. Treatment with this compound can slow the growth of implanted tumors in mouse model systems. Nevertheless, in one tumor implant study the compound was found in only very low concentrations in implanted tumor. PS-341 does not effect hematopoietic stem cell function in mice.

Some evidence suggests the theoretical possibility that inhibition of the proteasome could alter the concentration of PrP proteins in the cytosol of neurons. It is conceivable that this could exacerbate spongiform encephalopathies or even initiate them by inducing the formation of PrP^{sc} proteins. PS-341 is found only in very low concentrations in the brains of animals given a single radiolabeled dose. This finding may mitigate any potential problem with the formation of PrP^{sc}.

Its pattern of cellular resistance, its unusual tissue distribution, its unusually steep dose response curve, and its spectrum of toxic symptoms all suggest that PS-341 may cause toxicity through mechanisms other than simple inhibition of the 20S-proteasome. As it is a substituted dipeptide, its structure also suggests this possibility. PS-341 may interact with other cellular sites. This possibility has been little considered or examined. The mechanism by which it may exert any anti-tumor activity remains unclear.

II. SAFETY PHARMACOLOGY:

Reviewed by Sandi L. Verbois, Ph.D.

1. 6837- 113: PS- 341: Cardiovascular Effects after Intravenous Administration in Telemetered Cynomolgus Monkeys. Volume 4.2.1.3.1.

Conducting Laboratory and Location: (redacted)
Date of Study initiation: August 6, 1997
GLP Compliance: NO
Species and strain: Cynomolgus Monkeys
#/sex/group: 1/sex
Weight: 3.1 kg (male); 2.4 kg (female)
Drug, lot #, purity: The test material, PS-341, Lot No. 5; (redacted) pure. It was received at (redacted) on June 11, 1997. An expiration of June 1998 was provided.
Dose/Route/Volume/Duration: 0.2 mg/kg (2.4 mg/m^2) PS-341on Day 1 (Phase 1). A second dose at 0.3 mg/kg (3.6mg/m^2) was administered on Day 32 (Phase 2). The dose volume on each day was 1 mL/kg.
Observations:

Mortality /Moribundity- On Day 1, animals were observed pre-dose and approximately 1, 2, 4, and 6 hours postdose; observations of abnormal findings or an indication of normal were recorded. On Day 32, animals were observed pre-dose and were monitored via a video monitor after dosing; abnormal findings were recorded as they were observed. On nondosing days; abnormal findings were recorded twice daily (a.m. and p,m.)

Body weights -weekly

Food consumption- daily

Physical examinations- prior to treatment and day 2 (respiration and rectal temp)

Electrocardiographic (Lead II)/blood pressure measurements- recorded telemetrically before each dose (6 hours before first dose, 1 hour before second dose) and continuously for up to 24 hours after dosing of 0.2 mg/kg and for 12 hours after dosing of 0.3 mg/kg. An electrocardiogram (ECG) and blood pressure telemetry transmitter (redacted) was implanted into the abdominal cavity of each animal eight days before initiation of treatment. The ECG leads were arranged in a Lead II configuration; the blood pressure catheter was placed in a femoral artery.

Scheduled Necropsy- No scheduled necropy, to be returned to stock..

Mortality and Clinical Signs:

	Male			Female		
	0.2 mg/kg	**Interdose Interval**	**0.3 mg/kg**	**0.2 mg/kg**	**Interdose Interval**	**0.3 mg/kg**
Clinical Observations	None	None	Vomiting up to 6 hours PD*	Vomiting 6 hours PD to AM day 2	Menstruating Days 3, 4, 7, 8	Vomiting 4.5 – 11 hours PD
Body Weight	None	None	None	None	None	None
Food Consumption	None	None	None	None	None	None
Respiration	None	None	None	None	None	None
Body Temperature	None	None	None	None	None	None

*PD- Post-dose

Comment: Both animals were sacrificed by an overdose of sodium pentobarbital approximately 12 hours after dosing on Day 32. The animals were refrigerated overnight and necropsies were done the next day. The summary of safety pharmacology in the non-clinical overview (2.4.2.6 'Safety

Pharmacology Studies' on page 11), indicates that in one study animals were sacrificed moribund 14 hours following dosing. Reponses from the sponsor clarified that both animals were sacrificed moribund. No planned sacrifice was planned for these animals.

Pathology Data:

	Male	Female
Injection Site	Diffusely red; within subcutaneous tissue surrounding saphenous veins	Red Focus; within subcutaneous tissue surrounding saphenous veins: 0.4 cm and 1.2 cm (Left/Right)
Abdominal cavity	Thickened; connective tissue surrounding telemetry device.	-
Lung	Mottled, all left lobes	-
Stomach	Mottled	-

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Additionally, adrenal (2), kidney (2), cecum, lesion, colon, liver, duodenum, heart, lymph node (mesenteric), ileum, spleen, jejunum, and thymus samples were taken during necropsy, however no gross changes were noted in these tissues.

The company asserts that, **"There were no remarkable changes in mean blood pressure, heart rate, or electrocardiographic measurements after administration of 0.2 or 0.3 mg/kg"** (2.4 and 3.6 mg/m^2), however it appears as though there is a consistent pattern towards increasing HR and decreasing MAP following both doses, as seen in the graphs below.